PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :	(11) International Publication Number:	WO 93/07883
A61K 31/70, C07H 21/00 2122030 A1	(43) International Publication Date:	29 April 1993 (29.04.93)

(21) International Application Number: PCT/US92/09196

(22) International Filing Date: 23 October 1992 (23.10.92)

(30) Priority data:
782,374   24 October 1991 (24.10.91)   US

(60) Parent Application or Grant
   (63) Related by Continuation
      US   782,374 (CIP)
      Filed on   24 October 1991 (24.10.91)

(71) Applicant (for all designated States except US): ISIS PHARMACEUTICALS, INC. [US/US]; 2280 Faraday Avenue, Carlsbad, CA 92008 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only) : MANOHARAN, Muthiah [IN/US]; 2340 Hosp Way, #119, Carlsbad, CA 92008 (US). COOK, Philip, Dan [US/US]; 6430 La Paloma Street, Carlsbad, CA 92009 (US). BENNETT, Clarence, Frank [US/US]; 6553 Courte Christo, Carlsbad, CA 92008 (US).

(74) Agents: CALDWELL, John, W. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris, One Liberty Place - 46th Floor, Philadelphia, PA 19103-7301 (US).

(81) Designated States: AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
   With international search report.
   Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

2/220 30

(54) Title: DERIVATIZED OLIGONUCLEOTIDES HAVING IMPROVED UPTAKE AND OTHER PROPERTIES

(57) Abstract

    Linked nucleosides having at least one functionalized nucleoside that bears a substituent such as a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, a pyrene, a hybrid photonuclease/intercalator, or an aryl azide photo-crosslinking agent exhibit increased cellular uptake and other properties. The substituent can be attached at the 2'-position of the functionalized nucleoside via a linking group. If at least a portion of the remaining linked nucleosides are 2'-deoxy-2'-fluoro, 2'-O-methoxy, 2'-O-ethoxy, 2'-O-propoxy, 2'-O-aminoalkoxy or 2'-O-allyloxy nucleosides, the substituent can be attached via a linking group at any of the 3' or the 5' positions of the nucleoside or on the heterocyclic base of the nucleoside or on the inter-nucleotide linkage linking the nucleoside to an adjacent nucleoside.

1

DERIVATIZED OLIGONUCLEOTIDES HAVING IMPROVED UPTAKE
AND OTHER PROPERTIES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is related to Canadian patent application 2,073,500 filed January 11, 1991, Canadian patent application 2,089,377 filed on August 12, 1991 and Canadian patent application 2,140,428, filed on July 20, 1993.

FIELD OF THE INVENTION

This application is directed to sequence specific
10   oligonucleotides that include functionalized nucleosides having substituents such as steroids, reporter molecules, reporter enzymes, non-aromatic lipophilic molecules, peptides, or proteins attached via linking groups.

BACKGROUND OF THE INVENTION

Messenger RNA (mRNA) directs protein synthesis. Antisense methodology is the complementary hybridization of relatively short oligonucleotides to mRNA or DNA such that the normal, essential functions of these intracellular nucleic acids are disrupted. Hybridization is the sequence-specific
20   hydrogen bonding of oligonucleotides to RNA or single-stranded DNA via complementary Watson-Crick base pairs.

A

2122030   2

        The naturally occurring events that provide the
disruption of the nucleic acid function, discussed by Cohen
in *Oligonucleotides: Antisense Inhibitors of Gene Expression*,
CRC Press, Inc., Boca Raton, Fl (1989), are thought to be of
5 two types.   The first, hybridization arrest, denotes a
terminating event in which the oligonucleotide inhibitor binds
to the target nucleic acid and thus prevents, by simple steric
hindrance, the binding of essential proteins, most often
ribosomes, to the nucleic acid.   Methyl phosphonate
10 oligonucleotides (see, e.g., Miller, et al., *Anti-Cancer Drug
Design* 1987, *2*, 117) and α-anomer oligonucleotides, the two
most extensively studied antisense agents, are thought to
disrupt nucleic acid function by hybridization arrest.
        The second type of terminating event for antisense
15 oligonucleotides involves the enzymatic cleavage of targeted
RNA by intracellular RNase H.   A 2'-deoxyribofuranosyl
oligonucleotide or oligonucleotide analog hybridizes with the
targeted RNA to form a duplex that activates the RNase H
enzyme to cleave the RNA strand, thus destroying the normal
20 function of the RNA.   Phosphorothioate oligonucleotides
provide the most prominent example of antisense agents that
operate by this type of antisense terminating event.
        Considerable research is being directed to the
application of oligonucleotides and oligonucleotide analogs
25 as antisense agents for diagnostics, research reagents, and
therapeutics.   At least for therapeutic purposes, the
antisense oligonucleotides and oligonucleotide analogs must
be transported across cell membranes or taken up by cells to
express activity.   One method for increasing membrane or
30 cellular transport is by the attachment of a pendant
lipophilic group.
        Ramirez, et al., *J. Am. Chem. Soc.* 1982, *104*:, 5483,
introduced the phospholipid group 5'-O-(1,2-di-O-myristoyl-sn-
glycero-3-phosphoryl) into the dimer TpT independently at the
35 3' and 5' positions.   Subsequently Shea, et al., *Nuc. Acids
Res.* 1990, *18*, 3777, disclosed oligonucleotides having a 1,2-
di-O-hexyldecyl-rac-glycerol group linked to a 5'-phosphate

on the 5'-terminus of the oligonucleotide. Certain of the

Shea, et al authors disclosed these and other compounds in

patent application WO 90/10448 published on September 20th,

1990. Another glucosyl phospholipid was disclosed by Guerra,

et al., *Tetrahedron Letters* 1987, 28, 3581.

In other work, a cholesteryl group was attached to

the inter-nucleotide linkage between the first and second

nucleotides (from the 3' terminus) of an oligonucleotide.

This work is disclosed in United States Patent No. 4,958,013

10   and by Letsinger, et al., *Proc. Natl. Acad. Sci. USA* 1989, 86,

6553. The aromatic intercalating agent anthraquinone was

attached to the 2' position of a sugar fragment of an

oligonucleotide as reported by Yamana, et al., *Bioconjugate*

Chem. 1990, 1, 319.

Lemairte, et al., *Proc. Natl. Acad. Sci. USA* 1986,

84, 648 and Leonetti, et al., *Bioconjugate Chem.* 1990, 1, 149,

disclose modifying the 3' terminus of an oligonucleotide to

include a 3'-terminal ribose sugar moiety. Poly(L-lysine) was

linked to the oligonucleotide via periodate oxidation of this

20   terminal ribose followed by reduction and coupling through a

N-morpholine ring. Oligonucleotide-poly(L-lysine) conjugates

are described in European published patent application

0,251,283 A 2 published on January 7, 1988 wherein the lysine

residue was coupled to a 5' or 3' phosphate of the 5' or 3'

terminal nucleotide of the oligonucleotide. A disulfide

linkage has also been utilized at the 3' terminus of an

oligonucleotide to link a peptide to the oligonucleotide, as

described by Corey, et al., *Science* 1987, 238, 1401;

Zukermann, et al., *J. Am. Chem. Soc.* 1988, 110, 1614; and

Corey, et al., *J. Am. Chem. Soc.* 1989, 111, 8524.

Nelson, et al., *Nuc. Acids Res.* 1989, 17, 7187

describe a linking reagent for attaching biotin to the 3'-

terminus of an oligonucleotide. This reagent, N-Fmoc-O-DMT-3-

amino-1,2-propanediol, is commercially available from Clontech

Laboratories (Palo Alto, CA) under the name 3'-Amine on and

from Glen Research Corporation (Sterling, VA) under the name

10 3'-Amino-Modifier*. This reagent was also utilized to link a

peptide to an oligonucleotide, as reported by Judy, et al.,

Tetrahedron Letters 1992, 32, 879. A similar commercial

reagent (actually a series of linkers having various lengths

of polymethylene connectors) for linking to the 5'-terminus of

an oligonucleotide is 5'-Amino-Modifier* C6, also from Glen

Research Corporation. These compounds or similar ones were

utilized by Krieg, et al., *Antisense Research and Development*

1991, 1, 161 to link fluorescein to the 5'-terminus of an

oligonucleotide. Other compounds of interest have also been

20 linked to the 3'-terminus of an oligonucleotide. Asseline, et

al., *Proc. Natl. Acad. Sci. USA* 1984, 81, 3297 described

linking acridine on the 3'-terminal phosphate group of an poly

(Tp) oligonucleotide via a polymethylene linkage.

Haralambidis, et al., *Tetrahedron Letters* 1987, 28, 5199

reported building a peptide on a solid state support and then

linking an oligonucleotide to that peptide via the 3' hydroxyl

group of the 3' terminal nucleotide of the oligonucleotide.

A

63189-337.

Chollet, *Nucleosides & Nucleotides* 1990, 9, 957 attached an
Aminolink 2 (Applied Biosystems, Foster City, CA) to the 5'
terminal phosphate of an oligonucleotide. They then used the
bifunctional linking group SMPB (Pierce Chemical Co.,
Rockford, Il) to link an interleukin protein to the
oligonucleotide.

An EDTA iron complex has been linked to the 5
position of a pyrimidine nucleoside as reported by Dreyer, et
al., *Proc. Natl. Acad. Sci. USA* 1985, 82, 968. Fluorescein
has been linked to an oligonucleotide in the same manner, as
reported by Haralambidis, et al., *Nucleic Acid Research* 1987,
15, 4857 and biotin in the same manner as described in PCT
application WO 086/02929 published on May 22, 1986.
Fluorescein, biotin and pyrene were also linked in the same
manner as reported by Telser, et al., *J. Am. Chem. Soc.* 1989,
111, 6966. A commercial reagent, Amino-Modifier-dT* from Glen
Research Corporation, can be utilized to introduce pyrimidine
nucleotides bearing similar linking groups into
oligonucleotides.

* Trade-mark

5

Cholic acid linked to EDTA for use in radioscintigraphic imaging studies was reported by Betebenner, et al., *Bioconjugate Chem.* 1991, 2, 117; however, it is not known to link cholic acid to nucleosides, nucleotides or
5 oligonucleotides.

OBJECTS OF THE INVENTION

It is an object of this invention to provide sequence-specific oligonucleotides having improved transfer across cellular membranes.
10 It is a further object of this invention to provide improvements in research and diagnostic methods and materials for assaying bodily states in animals, especially disease states.

It is an additional object of this invention to provide
15 therapeutic and research materials having improved transfer and uptake properties for the treatment of diseases through modulation of the activity of DNA or RNA.

BRIEF DESCRIPTION OF THE INVENTION
20 In accordance with these and other objects evident from this specification, there are provided compounds that comprise a plurality of linked nucleosides wherein at least one of the nucleosides is functionalized at the 2'-position with a substituent such as, for example, a steroid molecule, a
25 reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, a hybrid photonuclease/intercalator, a pyrene, or an aryl azide photo-
30 crosslinking agent. Preferably, the substituent is connected to 2'-position using an intervening linking group.

In certain preferred embodiments of the invention, the substituents comprise a steroid molecule, biotin, a reporter enzyme or a fluorescein dye molecule. In these embodiments,
35 the steroid molecule is selected from the group consisting of cholic acid, deoxycholic acid, dehydrocholic acid, cortisone,

2122030   6

testosterone, cholesterol and digoxigenin with the most
preferred steroid molecule being cholic acid.   Preferred
reporter enzymes include horseradish peroxidase and alkaline
phosphatase.

5   In further preferred embodiments, the non-aromatic
lipophilic molecule attached to the 2'-position comprises an
alicyclic hydrocarbon, saturated or unsaturated fatty acid,
wax, terpenoid, or polyalicyclic hydrocarbon, including
adamantane and buckminsterfullerenes. Waxes according to the

10   invention include monohydric alcohol esters of fatty acids and
fatty diamides.   Buckminsterfullerenes include soccer ball-
shaped, cage molecules comprising varying numbers of
covalently bound carbon atoms.   Terpenoids include the C_{10}
terpenes, C_{20} sesquiterpenes, C_{30} diterpenes including vitamin

15   A (retinol), retinoic acid, retinal and dehydroretinol, C_{30}
triterpenes, C_{40} tetraterpenes   and   other   higher
polyterpenoids.

           In other preferred embodiments, peptides or proteins
attached   to   the   2'-position   comprise   sequence-specific
20   peptides   and   sequence-specific   proteins,   including
phosphatases, peroxidases and nucleases.

           Preferred linking molecules of the invention comprise
Ω-aminoalkoxy linkers, Ω-aminoalkylamino linkers, heterobi-
functional   linkers   or   homobifunctional   linkers.   A par-
25   ticularly preferred linking molecule of the invention is a 5-
aminopentoxy group.

           In preferred embodiments of the invention at least a
portion of the linked nucleosides are 2'-deoxy-2'-fluoro, 2'-
methoxy, 2'-ethoxy, 2'-propoxy, 2'-aminoalkoxy or 2'-allyloxy
30   nucleosides.   In other preferred embodiments of the invention
the   linked   nucleosides   are   linked   with   phosphorothioate
linking groups.

           The invention also provides compounds that have a
plurality of linked nucleosides.   In preferred embodiments,
35   at least one of the nucleosides is: (1) a 2'-functionalized
nucleoside having cholic acid linked to its 2'-position; (2)
a heterocyclic base functionalized nucleoside having cholic

acid linked to its heterocyclic base; (3) a 5' terminal nucleoside having cholic acid linked to its 5'-position; (4) a 3' terminal nucleoside having cholic acid linked to its 3'-position; or (5) an inter-strand nucleoside having cholic acid

5   linked to an inter-stand linkage linking said inter-strand nucleoside to an adjacent nucleoside.

In certain embodiments of the invention having linked nucleosides, at least one linked nucleosides bears a $2'$-deoxy'$-2'$-fluoro, $2'$-O-C_1-C_{20}-alkyl, $2'$-O-C_2-C_{20}-alkenyl, $2'$-O-

10   C_2-C_{20}-alkynyl, $2'$-S-C_1-C_{20}-alkyl, $2'$-S-C_2-C_{20}-alkenyl, $2'$-S-C_2-C_{20}-alkynyl, $2'$-NH-C_1-C_{20}-alkyl, $2'$-NH-C_2-C_{20}-alkenyl, or $2'$-NH-C_2-C_{20}-alkynyl substituent.

Further in accordance with the invention there is provided a method of increasing cellular uptake of a compound

15   having a plurality of linked nucleosides that includes contacting an organism with a compound where the compound includes at least one nucleoside functionalized at the $2'$-position with a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide,

20   a protein, a water soluble vitamin, and a lipid soluble vitamin. The compound can be included in a composition that further includes an inert carrier for the compound.

The invention also provides a method for enhancing the binding affinity and/or stability of an antisense

25   oligonucleotide comprising functionalizing the oligonucleotide with a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, and a lipid soluble vitamin.

2122030   8

DETAILED DESCRIPTION OF THE INVENTION

Antisense therapeutics can be practiced in a variety
of organisms ranging from unicellular prokaryotic and eukaryo-
tic organisms to multicellular eukaryotic organisms.   Any
5   organism that utilizes DNA-RNA transcription or RNA-protein
translation as a fundamental part of its hereditary, metabolic
or cellular control is susceptible to antisense therapeutics
and/or prophylactics.   Seemingly diverse organisms such as
bacteria, yeast, protozoa, algae, all plant and all higher
10   animal forms, including warm-blooded animals, can be treated
by antisense therapy.   Further, since each of the cells of
multicellular   eukaryotes   also   includes   both   DNA-RNA
transcription and RNA-protein translation as an integral part
of its cellular activity, antisense therapeutics and/or
15   diagnostics can also be practiced on such cellular popula-
tions.   Furthermore, many of the organelles, e.g., mito-
chondria and chloroplasts, of eukaryotic cells also include
transcription and translation mechanisms.   As such, single
cells, cellular populations or organelles can also be included
20   within the definition of organisms that are capable of being
treated with antisense therapeutics or diagnostics.   As used
herein, therapeutics is meant to include both the eradication
of a disease state, killing of an organism, e.g., bacterial,
protozoan or other infection, or control of erratic or harmful
25   cellular. growth or expression.

While we do not wish to be bound by any particular
theory, it is believed that the presence of many nuclear
proteins in the nucleus is due to their selective entry
through the nuclear envelope rather than to their selective
30   retention within the nucleus after entry.   By this mechanism,
the nucleus is able to selectively take up certain proteins
and not others.   The uptake is based upon the sequence of the
peptide or protein, which provides a selective signal sequence
that allows accumulation of the peptide or protein in the
35   nucleus.   One such peptide signal sequence is found as part
of the SV40 large T-antigen. *See, e.g.,* Dingwell, *et al. Ann.
Rev. Cell Bio.* **1986,** *2,* 367; Yoneda, *et al., Experimental Cell*

Research 1987, *170*, 439; and Wychowski, et al., *J. Virol.*
1986, *61*, 3862.

According to the present invention a substituent such
as a steroid molecule, a reporter molecule, a non-aromatic
5   lipophilic molecule, a reporter enzyme, a peptide, a protein,
a water soluble vitamin, a lipid soluble vitamin, an RNA
cleaving complex, a metal chelator, a porphyrin, an alkylator,
a hybrid photonuclease/intercalator, or an aryl azide photo-
crosslinking agent is attached to at least one nucleoside in
10 an antisense diagnostic or therapeutic agent to assist in the
transfer of the antisense therapeutic or diagnostic agent
across cellular membranes.   Such antisense diagnostic or
therapeutic agent is formed from a plurality of linked
nucleosides of a sequence that is "antisense" to a region of
15 an RNA or DNA that is of interest.   Thus, one or more
nucleoside of the linked nucleosides are "functionalized" to
include a substituent linked to the nucleoside via a linking
group.   For   the   purposes   of   identification,   such
functionalized   nucleosides   can   be   characterized   as
20 substituent-bearing   (e.g.,   steroid-bearing)   nucleosides.
Linked   nucleosides   having   at   least   one   functionalized
nucleoside   within   their   sequence   demonstrate   enhanced
antisense activity when compared to linked nucleoside that do
not contain functionalized nucleoside. These "functionalized"
25 linked   nucleosides   further   demonstrate   increased   transfer
across cellular membranes.

For the purposes of this invention. the terms "reporter
molecule" and "reporter enzyme" include molecules or enzymes
having physical or chemical properties that allow them to be
30 identified in gels, fluids, whole cellular systems, broken
cellular systems, and the like utilizing physical properties
such as spectroscopy, radioactivity, colorimetric assays,
fluorescence, and specific binding. Steroids include chemical
compounds that contain a perhydro-1,2-cyclopentanophenanthrene
35 ring system.   Proteins and peptides are utilized in their
usual sense as polymers of amino acids. Normally peptides are
amino acid polymers that contain a fewer amino acid monomers

per unit molecule than proteins. Non-aromatic lipophilic
molecules include fatty acids, esters, alcohols and other
lipid molecules, as well as synthetic cage structures such as
adamantane and buckminsterfullerenes that do not include
5   aromatic rings within their structure.

Particularly useful as steroid molecules are the bile
acids, including cholic acid, deoxycholic acid and dehydro-
cholic acid. Other useful steroids are cortisone,
digoxigenin, testosterone, cholesterol and cationic steroids
10   such as cortisone having a trimethylaminomethyl hydrazide
group attached via a double bond at the 3 position of the
cortisone rings. Particularly useful reporter molecules are
biotin and fluorescein dyes. Particularly useful non-aromatic
lipophilic molecules are alicyclic hydrocarbons, saturated and
15   unsaturated fatty acids, waxes, terpenes, and polyalicyclic
hydrocarbons, including adamantane and buckminsterfullerenes.
Particularly useful reporter enzymes are alkaline phosphatase
and horseradish peroxidase. Particularly useful peptides and
proteins are sequence-specific peptides and proteins,
20   including phosphodiesterase, peroxidase, phosphatase, and
nuclease proteins. Such peptides and proteins include SV40
peptide, RNase A, RNase H and Staphylococcal nuclease.
Particularly useful terpenoids are vitamin A, retinoic acid,
retinal, and dehydroretinol.
25   Vitamins according to the invention generally can be
classified as water soluble or lipid soluble. Water soluble
vitamins include thiamine, riboflavin, nicotinic acid or
niacin, the vitamin B_6 pyridoxal group, pantothenic acid,
biotin, folic acid, the B_{12} cobamide coenzymes, inositol,
30   choline and ascorbic acid. Lipid soluble vitamins include the
vitamin A family, vitamin D, the vitamin E tocopherol family
and vitamin K (and phytols). The vitamin A family, including
retinoic acid and retinol, are absorbed and transported to
target tissues through their interaction with specific
35   proteins such as cytosol retinol-binding protein type II
(CRBP-II), Retinol-binding protein (RBP), and cellular
retinol-binding protein (CRBP). These proteins, which have

been found in various parts of the human body, have molecular weights of approximately 15 kD.   They have specific interactions with compounds of vitamin-A family, especially, retinoic acid and retinol.

5   The vitamin A family of compounds can be attached to oligonucleotides via acid or alcohol functionalities found in the various family members. For example, conjugation of an N-hydroxy succinimide ester of an acid moiety of retinoic acid to an amine function on a linker pendant to an oligonucleotide

10   resulted in linkage of vitamin A compound to the oligonucleotide via an amide bond.   Also, retinol was converted to its phosphoramidite, which is useful for 5' conjugation.

α-Tocopherol (vitamin E) and the other tocopherols

15   (beta through zeta) can be conjugated to oligonucleotides to enhance uptake because of their lipophilic character. Also, the lipophilic vitamin, vitamin D, and its ergosterol precursors can be conjugated to oligonucleotides through their hydroxyl groups by first activating the hydroxyls groups to,

20   for example, hemisuccinate esters.   Conjugation then is effected to an aminolinker pendant from the oligonucleotide. Other vitamins that can be conjugated to oligonucleotide aminolinkers through hydroxyl groups on the vitamins include thiamine, riboflavin, pyridoxine, pyridoxamine, pyridoxal,

25   deoxypyridoxine.   Lipid soluble vitamin K's and related quinone-containing compounds can be conjugated via carbonyl groups on the quinone ring.   The phytol moiety of vitamin K may also serve to enhance bind of the oligonucleotides to cells.

30   Pyridoxal (vitamin B_6) has specific B_6-binding proteins. The role of these proteins in pyridoxal transport has been studied by Zhang and McCormick, *Proc. Natl. Acad. Sci. USA*, **1991** 88, 10407. Zhang and McCormick also have shown that a series of N-(4'-pyridoxyl)amines, in which several

35   synthetic amines were conjugated at the 4'-position of pyridoxal, are able to enter cells by a process facilitated by the B6 transporter. They also demonstrated the release of

these synthetic amines within the cell.   Other pyridoxal
family members include pyridoxine, pyridoxamine, pyridoxal
phosphate, and pyridoxic acid.   Pyridoxic acid, niacin,
pantothenic acid, biotin, folic acid and ascorbic acid can be
5 conjugated to oligonucleotides using N-hydroxysuccinimide
esters that are reactive with aminolinkers located on the
oligonucleotide, as described above for retinoic acid.

Other groups for modifying antisense properties include
RNA cleaving complexes, pyrenes, metal chelators, porphyrins,
10 alkylators, hybrid intercalator/ligands and photo-crosslinking
agents.   RNA cleavers include o-phenanthroline/Cu complexes
and Ru(bipyridine)$_3^{2+}$ complexes.   The Ru(bpy)$_3^{2+}$ complexes
interact with nucleic acids and cleave nucleic acids photo-
chemically.   Metal chelators are include EDTA, DTPA, and o-
15 phenanthroline.   Alkylators   include   compounds   such   as
iodoacetamide.   Porphyrins include porphine, its substituted
forms, and metal complexes.   Pyrenes include pyrene and other
pyrene-based carboxylic acids that could be conjugated using
the similar protocols.

20   Hybrid   intercalator/ligands   include   the
photonuclease/intercalator   ligand   6-[[[9-[[6-(4-nitro-
benzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoyl-
pentafluorophenyl ester.   This compound has two noteworthy
features: an acridine moiety that is an intercalator and a p-
25 nitro benzamido group that is a photonuclease.

Photo-crosslinking agents include aryl azides such as,
for example, N-hydroxysucciniimidyl-4-azidobenzoate (HSAB) and
N-succinimidyl-6(-4'-azido-2'-nitrophenyl-amino)hexanoate
(SANPAH).   Aryl azides conjugated to oligonucleotides effect
30 crosslinking with nucleic acids and proteins upon irradiation,
They also crosslink with carrier proteins (such as KLH or
BSA), raising antibody against the oligonucleotides.

A variety of linking groups can be used to connect the
substituents of the invention to nucleosides, nucleotides,
35 and/or oligonucleotides.   Certain linking groups, such as n-
aminoalkoxy moieties and n-aminoalkylamino moieties, are
particularly useful for linking steroid molecules or reporter

molecules to the 2'-position of a nucleoside. Many linking groups are commercially available, including heterobifunctional and homobifunctional linking moieties available from the Pierce Co. (Rockford, Il).

5 Heterobifunctional and homobifunctional linking moieties are particularly useful in conjunction with the n-aminoalkoxy and n-aminoalkylamino moieties to form extended linkers that connect peptides and proteins to nucleosides. Other commercially available linking groups are 5'-Amino-Modifier

10 C6 and 3'-Amino-Modifier reagents, both available from Glen Research Corporation (Sterling, VA). 5'-Amino-Modifier C6 is also available from ABI (Applied Biosystems Inc., Foster City, CA) as Aminolink-2, and 3'-Amino-Modifier is also available from Clontech Laboratories Inc. (Palo Alto, CA). A nucleotide

15 analog bearing a linking group pre-attached to the nucleoside is commercially available from Glen Research Corporation under the tradename "Amino-Modifier-dT." This nucleoside-linking group reagent, a uridine derivative having an [N(7-trifluoro-acetylaminoheptyl)3-acrylamido] substituent group at the 5

20 position of the pyrimidine ring, is synthesized generally according to Jablonski, et al., *Nucleic Acid Research* 1986, *14*, 6115. It is intended that the nucleoside analogs of the invention include adenine nucleosides functionalized with linkers on their N6 purine amino groups, guanine nucleosides

25 functionalized with linkers at their exocyclic N2 purine amino groups, and cytosine nucleosides functionalized with linkers on either their N4 pyrimidine amino groups or 5 pyrimidine positions.

Sequence-specific linked nucleosides of the invention

30 are assembled on a suitable DNA synthesizer utilizing either standard nucleotide precursors or nucleotide precursors that already bear linking moieties. Once synthesis of the sequence-specific linked nucleosides is complete, a substituent can be reacted with the linking moiety. Thus, the

35 invention preferably first builds a desired linked nucleoside sequence by known techniques on a DNA synthesizer. One or

more of the linked nucleosides are then functionalized or derivatized with a selected substituent.

Canadian patent applications 2,073,500; 2,089,377 and 2,140,428 disclose that incorporation of, for example, a 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-allyl, 2'-O-aminoalkyl or 2'-deoxy-2'-fluoro groups on the nucleosides of an oligonucleotide enhance the hybridization properties of the oligonucleotide. These applications also disclose that oligonucleotides containing phosphorothioate backbones have

10   enhanced nuclease stability. The functionalized, linked nucleosides of the invention can be augmented to further include either or both a phosphorothioate backbone or a 2'-O-C_1-C_{20}-alkyl (e.g., 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl), 2'-O-C_2-C_{20}-alkenyl (e.g., 2'-O-allyl), 2'-O-C_2-C_{20}-alkynyl, 2'-S-C_1-C_{20}-alkyl, 2'-S-C_2-C_{20}-alkenyl, 2'-S-C_2-C_{20}-alkynyl, 2'-NH-C_1-C_{20}-alkyl (2'-O-aminoalkyl), 2'-NH-C_2-C_{20}-alkenyl, 2'-NH-C_2-C_{20}-alkynyl or 2'-deoxy-2'-fluoro group.

An oligonucleotide possessing an amino group at its 5'-terminus is prepared using a DNA synthesizer and then is

20   reacted with an active ester derivative of the substituent of the invention (e.g., cholic acid). Active ester derivatives are well known to those skilled in the art. Representative active esters include N-hydrosuccinimide esters, tetrafluorophenolic esters, pentafluorophenolic esters and pentachlorophenolic esters. For cholic acid, the reaction of

A

the amino group and the active ester produces an
oligonucleotide in which cholic acid is attached to the
5'-position through a linking group. The amino group at the
5'-terminus can be prepared conveniently utilizing the above-
noted 5'-Amino-Modifier C6 reagent.

Cholic acid can be attached to a 3'-terminal amino
group by reacting a 3'-amino modified controlled pore glass
(sold by Clontech Laboratories Inc., Palo Alto, CA), with a
cholic acid active ester.

10   Cholic acid can be attached to both ends of a linked
nucleoside sequence by reacting a 3',5'-diamino sequence with
the cholic acid active ester. The required oligonucleoside
sequence is synthesized utilizing the 3'-Amino-Modifier* and
the 5'-Amino-Modifier* C6 (or Aminolink-2*) reagents noted
above or by utilizing the above-noted 3'-amino modified
controlled pore glass reagent in combination with the
5'-Amino-Modifier* C2 (or Aminolink-2*) reagents.

In even further embodiments of the invention, an
oligonucleoside sequence bearing an aminolinker at the 2'-
20   position of one or more selected nucleosides is prepared using
a suitably functionalized nucleotide such as, for example,
5'-dimethoxytrityl-2'-O-(€-phthalimidylaminopentyl)-2'-
deoxyadenosine-3'-N,N-diisopropyl-cyanoethoxy phosphoramidite.
See, e.g., Manoharan, et al., *Tetrahedron Letters*, 1991, 34,
7171 and above-referenced Canadian patent application
2,073,500 and 2,089,377. The nucleotide or nucleotides are
attached to cholic acid or another substituent using an active

ester or a thioisocyanate thereof. This approach allows the
introduction of a large number of functional groups into a
linked nucleoside sequence. Indeed each of the nucleosides
can be so substituted.

In further functionalized nucleoside sequences of
the invention, the heterocyclic base of one or more
nucleosides is linked to a steroid molecule, a reporter
molecule, a non-aromatic lipophilic molecule, a reporter
enzyme, a peptide, a protein, a water soluble vitamin, a lipid
10   soluble vitamin, an RNA cleaving complex, a metal chelator, a
porphyrin, an alkylator, a hybrid photonuclease/intercalator,
or an aryl azide photo-crosslinking agent. Utilizing 5'-0-
dimethoxytrityl-5-[N(7-trifluoroacetylaminoheptyl)-3-
acrylamido]-2'-deoxyuridine-3'-0-(methyl N,N-diisopropyl)-
phosphoramide, as described by Jablonski, et al. above (also
commercially available from Glen Research), the desired
nucleoside, functionalized to incorporate a linking group on
its heterocyclic base, is incorporated into the linked
nucleoside sequence using a DNA synthesizer.

2122030   16

Conjugation (linking) of reporter enzymes, peptides,
and proteins to linked nucleosides is achieved by conjugation
of the enzyme, peptide or protein to the above-described amino
linking group on the nucleoside. This can be effected in
5 several ways.   A peptide- or protein- functionalized
nucleoside of the invention can be prepared by conjugation of
the peptide or protein to the nucleoside using EDC/sulfo-NHS
(i.e.,1-ethyl-3(3-dimethylaminopropylcarbodiimide/N-hydroxy-
sulfosuccinimide) to conjugate the carboxyl end of the
10 reporter enzyme, peptide, or protein with the amino function
of the linking group on the nucleotide. Further, a linked
nucleoside sequence of the invention can be prepared using
EDC/sulfo-NHS to conjugate a carboxyl group of an aspartic or
glutamic acid residue in the reporter enzyme, peptide or
15 protein to the amino function of a linked nucleoside sequence.

Preferably a reporter enzyme-, peptide-, protein-
functionalized linked nucleoside sequence can be prepared by
conjugation of the reporter enzyme, peptide or protein to the
nucleoside sequence via a heterobifunctional linker such as
20 m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (MBS) or
succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate
(SMCC) to link a thiol function on the reporter enzyme,
peptide or protein to the amino function of the linking group
on nucleoside sequence. By this mechanism, an oligonucleo-
25 side-maleimide conjugate is formed by reaction of the amino
group of the linker on the linked nucleosides with the MBS or
SMCC maleimide linker. The conjugate is then reacted with
peptides or proteins having free sulfhydryl groups.

In a second preferred method, a reporter enzyme-,
30 peptide-, protein-functionalized linked nucleoside sequence
can be prepared by conjugation of the peptide or protein to
the sequence using a homobifunctional linker such as
disuccinimidyl suberate (DSS) to link an amino function on the
peptide or protein to the amino group of a linker on the
35 sequence. By this mechanism, an oligonucleoside-succinimidyl
conjugate is formed by reaction of the amino group of the
linker on the nucleoside sequence with a disuccinimidyl sub-

erate linker. The disuccinimidyl suberate linker couples with
the amine linker on the sequence to extend the size of the
linker. The extended linker is then reacted with amine groups
such as, for example, the amine of lysine or other available
5   N-terminus amines, on reporter enzymes, peptides and proteins.

    Additional objects, advantages, and novel features of
this invention will become apparent to those skilled in the
art upon examination of the following examples, which are not
intended to be limiting.

10   For   the   following   examples,   anhydrous
dimethylformamide, cholic acid and N-hydroxysuccinimide were
purchased from Aldrich Chemical Co. (Milwaukee, WI), ethyl-3-
(3-dimethylamino)propylcarbodiimide (EDAC or EDC) was obtained
from JBL Scientific (San Luis Obispo, CA) as the free base
15   under the label EDAC or from Pierce (Rockford, Il) under the
label EDC, Aminolink-2 was purchased from ABI and 3'-Amino-
Modifier, 5'-Amino-Modifier C6 and Amino-Modifier dT reagents
were purchased from Glen Research Corporation. NMR Spectra
were run on a Varian Unity-400 instrument. Oligonucleotide
20   synthesis were performed on an Applied Biosystems 380 B or 394
DNA synthesizer following standard phosphoramidite protocols
using reagents supplied by the manufacturer. When modified
phophoramidites were used, a longer coupling time (10-15 min)
was employed. HPLC was performed on a Waters 600E instrument
25   equipped with a model 991 detector. Unless otherwise noted,
for analytical chromatography the following conditions were
employed: Hamilton PRP-1 column (15 x 2.5 cm); solvent A: 50mm
TEAA, pH 7.0; solvent B: 45mm TEAA with 80% CH_3CN; flow rate:
1.5ml/min; gradient: 5% B for the first 5 minutes, linear
30   (1%) increase in B every minute thereafter and for preparative
purposes: Waters Delta Pak C-4 column; flow rate: 5ml/min;
gradient: 5% B for the first 10 minutes, linear 1% increase
for every minute thereafter.

    All oligonucleotide sequences are listed in a standard
35   5' to 3' order from left to right.

EXAMPLE 1

Cholic Acid N-Hydroxysuccinimide Ester (Compound 1)

     Anhydrous DMF (150ml) was added to a mixture of cholic acid (4.09g, 15mmol) and N-hydroxysuccinimide (5.25g, 45

5 mmol). The mixture was stirred in the presence of nitrogen. EDAC (4ml, 25mmol) was then added and this mixture was then stirred overnight. The solution was then evaporated to a gum and partitioned between 1:1 ethyl acetate and 4% $NaHCO_3$ solution (pH 7.9) (100ml each). The organic layer was washed

10 with saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated to yield the title compound as a pale yellow foam (4.6g, 91%). ^{13}C NMR (DMSO-d_6) δ 12.27, 16.71, 22.58, 22.80, 25.42, 26.19, 27.20, 28.49, 30.41, 30.56, 34.36, 34.82, 34.82, 35.31, 39.09, 39.09, 41.38, 41.53, 45.84, 46.05, 66.25, 70.45,

15 71.03, 169.28 and 170.16.

EXAMPLE 2

Heterocyclic Base Cholic Acid End-Labeled Oligodeoxynucleotide

     An oligonucleotide of the sequence:

     Oligomer 1:   TTG CTT[*].CCA TCT TCC TCG TC

20 wherein T[*] represents a nucleotide functionalized to included a cholic acid linked via a linker to the heterocyclic base of a 2'-deoxyuridine nucleotide was prepared in a 1 μmol scale. Oligomer 1 is useful as an HPV antisense oligonucleotide.

     A. Preparation of Intermediate Linker

25   The linker of the structure -CH=CH-C(O)-NH-$(CH_2)_6$-NH-C(O)-CF_3 was introduced via a suitable protected and activated 2'-deoxyuridine phosphoramide intermediate obtained from Glen Research Corporation as Amino-Modifier-dT. The oligonucleotide bearing the linker thereon was deprotected and

30 purified by HPLC.

     B. Preparation of Cholic Acid Functionalized Oligonucleotide

     An aliquot of the linker bearing oligonucleotide of Example 2-A (approximately 100 O.D. units, 550 nmols) was

35 dissolved in 500 μl of 0.2M $NaHCO_3$ buffer and to this solution cholic acid N-hydroxysuccinimide ester (Compound 1, 75 mg, 149

μmols) was added and heated at 45°C overnight. It was then passed through a Sephadex G-25 (1.0 x 25 cm) column. Concentration of the oligonucleotide fractions to 2 ml followed by HPLC purification yielded the desired conjugate

5 wherein cholic acid is internally linked at C-5 of the heterocyclic base.

EXAMPLE 3

5'-Terminus Cholic Acid End-Labeled Oligodeoxynucleotide

A phosphorothioate oligonucleotide having cholic acid

10 attached to its 5'-terminus of the oligonucleotide sequence:
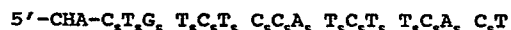
Oligomer 2:

5'-CHA-C$_s$T$_s$G$_s$ T$_s$C$_s$T$_s$ C$_s$C$_s$A$_s$ T$_s$C$_s$T$_s$ T$_s$C$_s$A$_s$ C$_s$T

wherein CHA represents cholic acid and the subscript "s" represents a phosphorothioate inter-nucleotide backbone

15 linkage was prepared.

A. Preparation of Intermediate Linker

The oligonucleotide sequence having a 5'-terminus amino group was synthesized on a 3 x 1.0 μmol scale in the standard manner on the DNA synthesizer utilizing phosphoramidite

20 methodology. The phosphorothioate intra-nucleotide backbone was formed using a phosphorothioate reagent (Beaucage reagent, i.e., 3H-1,2-benzodithioate-3-one 1,1-dioxide; see, Radhakrishnan, et al., J. Am. Chem. Soc. 1990, 112, 1253). The Aminolink-2 reagent was used at the last step of the

25 oligonucleotide synthesis. Deprotection with concentrated NH$_4$OH for 16 hrs at 55°C yielded the 5'-aminolinker-oligonucleotide.

B. Preparation of Cholic Acid Functionalized Oligonucleotide

30 The crude 5'-aminolinker-oligonucleotide of Example 3-A (100 O.D. units, approximately 600 nmols based on the calculated extinction coefficient of 1.6756 x 10^5 at 260 nm) was dissolved in freshly prepared NaHCO$_3$ buffer (500 μl, 0.2M, pH 8.1) and treated with a solution of cholic acid N-

35 hydroxysuccinimide ester (Compound 1, 75 mg, 149 μmols) dissolved in 200 μl of DMF. The reaction mixture was heated

at 45°C overnight. It was then passed through a Sephadex G-25
(1.0 x 25 cm) column. Concentration of the oligonucleotide
fractions to 2 ml followed by HPLC purification yielded 54 OD
units of the desired conjugate (54% yield). HPLC retention
5 times were: 37.42 for the unreacted oligonucleotide and any
failure sequences produced during oligonucleotide synthesis
and 54.20 for the final product.

EXAMPLE 4
3'-Terminus Cholic Acid End Labeled Oligodeoxynucleotide
10   **A.   3'-Terminus Cholic Acid Oligonucleotide**
        A phosphorothioate oligonucleotide having cholic acid
attached to its 3'-terminus of the oligonucleotide sequence:
        Oligomer 3:

        $C_sT_sG_s$ $T_sC_sT_s$ $C_sC_sA_s$ $T_sC_sC_s$ $T_sC_sT_s$ $T_sC_sA_s$ C_sT 3'-CHA

15 wherein CHA represents cholic acid and the subscript "s"
represents a phosphorothioate inter-nucleotide backbone
linkage was prepared.
        1.   Preparation of Intermediate Linker
        The oligonucleotide sequence having a 3'-terminus amino
20 group was synthesized using 3'-amino modifier controlled pore
glass (CPG) from Clontech Laboratories (Palo Alto, CA) as the
solid support. The above-noted Beaucage reagent was utilized
to form the phosphorothioate inter-nucleotide backbone. The
synthesis was conducted in a "Trityl-Off" mode. The resultant
25 solid support was deprotected with concentrated NH_4OH for 16
hrs at 55°C. Purification on a Sephadex G-25 column yielded
a 3'-amino functionalized phosphorothioate oligonucleotide of
the specified oligonucleotide sequence.
        2.   Preparation of Cholic Acid Functionalized
30 **Oligonucleotide**
        The crude oligonucleotide of Example 4-A-1 (50 O.D.
units, approximately 300 nmols) was reacted with cholic acid
N-hydroxysuccinimide ester (Compound 1, 40 mg) as per the
procedure of Example 3. HPLC retention times were 37.45 for
35 the starting oligonucleotide material and 51.68 for the
product.

B. 3′-Terminus Cholic Acid Oligonucleotide

A phosphorothioate oligonucleotide having cholic acid attached to its 3′-terminus and of the oligonucleotide sequence:

5   Oligomer 4:

$T_sG_sG_s$ $G_sA_sG_s$ $C_sC_sA_s$ $T_sA_sG_s$ $C_sG_sA_s$ G_sG_sC 3′-CHA

wherein CHA represents cholic acid and the subscript "s" represents a phosphorothioate inter-nucleotide backbone linkage was prepared in the same manner as for the

10 oligonucleotide of Example 4-A-2.

EXAMPLE 5

3′-Terminus Cholic Acid & 5′-Terminus Cholic Acid Di-End-Labeled Oligodeoxynucleotide

A phosphorothioate oligonucleotide having cholic acid

15 attached to both of the 3′-terminus and the 5′-terminus of the oligonucleotide sequence:

Oligomer 5:

5′-CHA $C_sT_sG_s$ $T_sC_sT_s$ $C_sC_sA_s$ $T_sC_sT_s$ $T_sC_sA_s$ C_sT 3′-CHA

wherein CHA represents cholic acid and the subscript "s"

20 represents a phosphorothioate inter-nucleotide backbone linkage was synthesized on a 3 x 1.0 μmol scale. Oligomer 5 has the same sequence as Oligomer 1 except for the cholic acid functionalization.

A. Preparation of Intermediate Linker

25   The oligonucleotide synthesis was conducted using a 3′-amino modified controlled pore glass (CPG) from Clontech Laboratories as the solid support. Oligonucleotide synthesis was conducted utilizing phosphoramidite synthetic methodology and the Beaucage reagent to form a phosphorothioate inter-

30 nucleotide backbone as per Example 4 above. Upon completion of the basic sequence and while still on the DNA synthesizer, Aminolink-2 reagent was used to introduce a 5′-terminus amino functionality on to the oligonucleotide. Deprotection with concentrated ammonium hydroxide and purification on a Sephadex

35 G-25 column yielded 3′,5′-diaminolinker oligonucleotide.

B.   Preparation   of   Cholic   Acid   Functionalized
Oligonucleotide

          The crude di-aminolinker oligonucleotide (50 O.D.
units, approximately 300 nmols, based on the calculated

5   extinction coefficient of 1.6756×10^5 at 260 nm) was
dissolved in freshly prepared $NaHCO_3$ buffer (500 μl, 0.2M, pH
8.1) and treated with a solution of cholic acid N-
hydroxysuccinimide ester (Compound 1, 50 mg, 98.6 μmols)
dissolved in 200 μl of DMF. The reaction mixture was heated

10   at 45°C overnight. It was then passed through a Sephadex G-25
(1.0 x 25 cm) column. The oligonucleotide fractions were
concentrated to 2 ml and purified by reverse phase HPLC.
Retention times were 37.76 for unreacted oligonucleotide,
51.65 for 3'-cholic acid conjugated oligonucleotide, 54.34 for

15   5'-cholic acid conjugated oligonucleotide and 58.75 for 3',5'-
di-cholic acid conjugated oligonucleotide. The 58.75 min.
product was desalted on a Sephadex G-25 column to yield 11 O.D
units (22%) of the desired product.

          EXAMPLE 6

20   **3'-Terminus   Cholic   Acid   or   5'-Terminus   Cholic   Acid
Functionalized, 2'-O-Methyl Derivatized Oligodeoxynucleotides**

          Phosphorothioate oligonucleotides having cholic acid
attached to either the 3'-terminus end or the 5'-terminus end
of the oligonucleotide sequence and further being uniformly

25   functionalized to include a 2'-O-methyl group on each of the
nucleotides of the oligonucleotide were synthesized. The
following   oligonucleotides   having   uniform   2'-O-methyl
substitutions were synthesized:

          Oligomer 6:   5'-CHA CCC AGG CUC AGA 3';

30   Oligomer 7:   5' CCC AGG CUC AGA 3'-CHA; and
          Oligomer 8:   5'-CHA GAG CUC CCA GGC 3'.

          A. Preparation of Intermediate Linker

          Synthesis of the intermediate 5' or 3'-aminolinker
oligonucleotide was conducted utilizing 2'-O-methyl phosphor-

35   amidite nucleotides available from Chemgenes Inc. (Needham,
MA) and phosphoramidite chemistry as per Examples 3 and 4

above, respectively. Each of the intermediate
oligonucleotides were deprotected in concentrated NH$_4$OH,
evaporated and de-salted on a Sephadex G-25 column.

B. Preparation of Cholic Acid Functionalized
5 Oligonucleotide

The resultant crude oligonucleotides from Example 6-A-1
(from 30 to 40 O.D. units, 250-350 nmols based on calculated
extinction coefficients of 1.1797 x 10^5, 1.1777 x 10^5 and
1.1481 x 10^5 at 260 nm, respectively for Oligomers 6, 7 and 8)
10 were dried and dissolved in 250 μl of 0.2M NaHCO$_3$ buffer and
treated with a solution of cholic acid N-hydroxysuccinimide
ester (Compound 1, from 30 to 40 mg, 60 to 80 μmols) dissolved
in 500 μl of 0.2 M NaHCO$_3$ buffer and 200 μl DMF and heated
between 40-45°C for 12-24 hrs. The reaction mixtures were
15 evaporated and dissolved in 2 ml of water and washed with 3
x 2 ml of ethyl acetate. The resultant aqueous solutions were
purified by reverse phase HPLC. For Oligomer 6 the HPLC
retention times were 34.65 for the starting oligonucleotide
material and 58.75 for the product; for Oligomer 7 the HPLC
20 retention times were 37.23 for the starting oligonucleotide
material and 55.32 for the product; and for Oligomer 8 the
HPLC retention times were 34.99 for the starting
oligonucleotide material and 56.98 for the product. The
products were evaporated and desalted on a Sephadex G-25
25 column. The yield averaged about 20% in each case.

EXAMPLE 7
Oligonucleotides Having 2'-Protected-Amine Terminating Linking
Group

A. Preparation of 5'-Dimethoxytrityl-2'-(O-Pentyl-N-
30 phthalimido)-2'-Deoxyadenosine Phosphoramidite (Compound 2)

To introduce a functionalization at the 2' position of
nucleotides within desired oligonucleotide sequences, 5'-
Dimethoxytrityl-2'-(O-pentyl-N-phthalimido)-2'-deoxyadenosine
phosphoramidite (Compound 2) was utilized to provide a linking
35 group attached to the 2' position of nucleotide components of
an oligonucleotide. Compound 2 was synthesized as per the

procedures of Canadian patent application 2,073,550
identified above starting from adenosine. Briefly this
procedure treats adenosine with NaH in DMF followed by
treatment with N-(5-bromopentyl)phthalimide. Further
5 treatment with $(CH_3)_3SiCl$, Ph-C(O)-Cl and NH_4OH yields N6-
benzyl protected 2'-pentyl-N-phthalimido functionalized
adenosine. Treatment with DIPA and CH_2Cl_2 adds a DMT blocking
group at the 5' position. Finally phosphitylation gives the
desired phosphoramidite compound, Compound 2. Compound 2 was
10 utilized in the DNA synthesizer as a 0.09M solution in
anhydrous CH_3CN. Oligonucleotide synthesis was carried out in
either an ABI 390B or 394 synthesizer employing the standard
synthesis cycles with an extended coupling time of 10 minutes
during coupling of Compound 2 into the oligonucleotide
15 sequence. Coupling efficiency of > 98% was observed for
Compound 2 coupling.

        B.   2'-Protected-Amine Linking Group Containing
Phosphodiester Oligonucleotides
        The following oligonucleotides having phosphodiester
20 inter-nucleotide linkages were synthesized:
        Oligomer 9:   5' TA*G 3';
        Oligomer 10:   5' CCA* G 3';
        Oligomer 11:   5' GGC TGA* CTG CG 3';
        Oligomer 12:   CTG TCT CCA* TCC TCT TCA CT; and
25   Oligomer 13:   CTG TCT CCA* TCC TCT TCA* CT
wherein A* represents a nucleotide functionalized to
incorporate a pentyl-N-phthalimido functionality. Oligomers
12 and 13 are antisense compounds to the E2 region of the
bovine papilloma virus-1 (BPV-1). Oligomers 12 and 13 have
30 the same sequence as Oligomer 3 except for the 2'
modification. The oligonucleotides were synthesized in either
a 10 µmol scale or a 3 x 1 µmol scale in the "Trityl-On" mode.
Standard deprotection conditions (30% NH_4OH, 55°C, 24 hr) were
employed. The oligonucleotides were purified by reverse phase
35 HPLC (Waters Delta-Pak *C_4 15 µm, 300A, 25x100 mm column
equipped with a guard column of the same material). They were
detritylated and further purified by size exclusion using a

* Trade-mark

A

63189-337

25

Sephadex G-25 column. NMR analyses by both proton and phosphorus NMR confirmed the expected structure for the Oligomers 9 and 10.

        C.   2'-Protected-Amine Linking Group Containing
5 Phosphorothioate Oligonucleotides

        The following oligonucleotides having phosphorothioate inter-nucleotide linkages were synthesized:

        Oligomer 14:

        $T_sT_sG_s$ $C_sT_sT_s$ $C_sC_sA*_s$ $T_sC_sT_s$ $T_sC_sC_s$ $T_sC_sG_s$ T_sC;
10   Oligomer 15:

        $T_sG_sG_s$ $G_sA_sG_s$ $C_sC_sA_s$ $T_sA_sG_s$ $C_sG_sA*_s$ G_sG_sC; and

        Oligomer 16:

        $T_sG_sG_s$ $G_sA*_sG_s$ $C_sC_sA*_s$ $T_sA*_sG_s$ $C_sG_sA*_s$ G_sG_sC

wherein A* represents a nucleotide functionalized to
15 incorporate a pentyl-N-phthalimido functionality and the subscript "s" represents a phosphorothioate inter-nucleotide backbone linkage. Oligomer 14 is an antisense compound directed to the E2 region of the bovine papilloma virus-1 (BPV-1). Oligomers 15 and 16 are antisense compounds to ICAM.
20 Oligomer 14 has the same sequence as Oligomer 3 except for the 2' modification whereas Oligomers 15 and 16 have the same sequence as Oligomer 4 except for the 2' modification. These oligonucleotides were synthesized as per the method of Example 7-B except during the synthesis, for oxidation of the
25 phosphite moieties, the Beaucage reagent (see Example 3 above) was used as a 0.24 M solution in anhydrous CH_3CN solvent. The oligonucleotides were synthesized in the "Trityl-On" mode and purified by reverse phase HPLC utilizing the purification procedure of Example 7-B.
30   D. 2'-O-Methyl Derivatized, 2'-Protected-Amine Linking Group Containing RNA Oligonucleotides

        The following oligonucleotides having 2'-O-methyl groups on each nucleotide not functionalized with a 2'-protected amine functionalization were synthesized:
35   Oligomer 17:   CCA A*GC CUC AGA; and

        Oligomer 18:   CCA GGC UCA GA*T

wherein A* represents a nucleotide functionalized to
incorporate a pentyl-N-phthalimido functionality and where the
remaining nucleotides except the 3'-terminus nucleotide are
each 2'-O-methyl derivatized nucleotides. The 3'-terminus

5   nucleotide in both Oligomers 17 and 18 is a 2'-deoxy
nucleotide. Both Oligomers 17 and 18 are antisense compounds
to the HIV-1 TAR region. The oligonucleotides were
synthesized as per the method of Example 6 utilizing Compound
2 for introduction of the nucleotides containing the pentyl-N-

10 phthalimido functionality and appropriate 2-O-methyl
phosphoramidite nucleotides from Chemgenes Inc. (Needham, MA)
for the remaining RNA nucleotides. The 3'-terminus terminal
2'-deoxy nucleotides were standard phosphoamidites utilized
for the DNA synthesizer. The oligonucleotides were

15 deprotected and purified as per the method of Example 7-B.

EXAMPLE 8
Functionalization Of Oligonucleotides At the 2' Position
        A. Functionalization with Biotin
            1. Single Site Modification

20   About 10 O.D. units (A_{260}) of Oligomer 12 (see Example
7) (approximately 60 nmols based on the calculated extinction
coefficient of 1.6756×10^5) was dried in a microfuge tube.
The oligonucleotide was dissolved in 200 μl of 0.2 M $NaHCO_3$
buffer and D-biotin-N-hydroxysuccinimide ester (2.5 mg, 7.3

25 μmols) (Sigma, St. Louis, MO) was added followed by 40 μl DMF.
The solution was let stand overnight. The solution was
applied to a Sephadex G-25 column (0.7 x 15 cm) and the
oligonucleotide fractions were combined. Analytical HPLC
showed nearly 85% conversion to the product. The product was

30 purified by HPLC (Waters 600E with 991 detector, Hamilton PRP-
1 column 0.7 x 15 cm; solvent A: 50 mM TEAA pH 7.0; B: 45 mM
TEAA with 80% acetonitrile: 1.5 ml flow rate: Gradient: 5% B
for first 5 mins., linear (1%) increase in B every minute
thereafter) and further desalted on Sephadex G-25 to give the

35 oligonucleotide:
            Oligomer 19:   CTG TCT CCA* TCC TCT TCA CT

wherein A* represents a nucleotide functionalized to incorporate a biotin functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

5   **2. Multiple Site Modification**

About 10 O.D. units (A_{260}) of Oligomer 13 (see Example 7, approximately 60 nmols) was treated utilizing the method of Example 8-A-1 with D-biotin-N-hydroxysuccinimide ester (5 mg) in 300 μl of 0.2 M NaHCO$_3$ buffer/ 50 μl DMF. Analytical

10   HPLC showed 65% of double labeled oligonucleotide product and 30% of single labeled products (from the two available reactive sites). HPLC and Sephadex G-25 purification gave the oligonucleotide:

Oligomer 20:   CTG TCT CCA* TCC TCT TCA* CT

15   wherein A* represents nucleotides functionalized to incorporate a biotin functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times for this product (and its accompanying singly labeled products) are shown in Table 1

20   below.

B. Functionalization with Fluorescein

1. Single Site Modification

A 1M Na$_2$CO$_3$/1M NaHCO$_3$ buffer (pH 9.0) was prepared by adding 1M NaHCO$_3$ to 1 M Na$_2$CO$_3$. 200 μl of this buffer was

25   added to 10 O.D. units of Oligomer 12 (see Example 7) in a microfuge tube. 10 mg of fluorescein-isocyanate in 500 μl DMF was added to give a 0.05 M solution. 100 μl of the fluorescein solution was added to the oligonucleotide solution in the microfuge tube. The tube was covered with aluminum

30   foil and let stand overnight. The reaction mixture was applied to a Sephadex G-25 column (0.7 x 20 cm) that had been equilibrated with 25% (v/v) ethyl alcohol in water. The column was eluted with the same solvent. Product migration could be seen as a yellow band well separated from dark yellow

35   band of the excess fluorescein reagent. The fractions showing absorption at 260 nm and 485 nm were combined and purified by HPLC as per the purification procedure of Example 8-A-1.

Analytical HPLC indicated 81% of the desired doubly functionalized oligonucleotide. The product was lyophilized and desalted on Sephadex to give the oligonucleotide:

Oligomer 21:   CTG TCT CCA* TCC TCT TCA CT

5   wherein A* represents a nucleotide functionalized to incorporate a fluorescein functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

10   **2. Multiple Site Modification**

10 O.D. units (A_{260}) of Oligomer 13 (from Example 7) was dissolved in 300 µl of the 1M Na_2HCO_3/ 1M Na_2CO_2 buffer of Example 8-B-1 and 200 µl of the fluorescein-isothiocyanate stock solution of Example 8-B-1 was added. The resulting

15 solution was treated as per Example 8-B-1. Analytical HPLC indicated 61% of doubly labeled product and 38% of singly labeled products. Work up of the reaction gave the oligonucleotide:

Oligomer 22:   CTG TCT CCA* TCC TCT TCA* CT

20 wherein A* represents nucleotides functionalized to incorporate a fluorescein functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the designated nucleotide. HPLC retention times are shown in Table 1 below.

25   **C. Functionalization with Cholic Acid**

          1. Single Site Modification

10 O.D. units (A_{260}) of Oligomer 12 (see Example 7) was treated with cholic acid-NHS ester (Compound 1, 5 mg, 9.9 µmols) in 200 µl of 0.2 M $NaHCO_3$ buffer/ 40 µl DMF. The

30 reaction mixture was heated for 16 hrs at 45°C. The product was isolated as per the method of Example 8-A-1. Analytical HPLC indicated > 85% product formation. Work up of the reaction gave the oligonucleotide:

Oligomer 23:   CTG TCT CCA* TCC TCT TCA CT

35 wherein A* represents a nucleotide functionalized to incorporate a cholic acid functionality linked via a 2'-O-pentyl-amino linking group to the 2' position of the

designated nucleotide. HPLC retention times are shown in
Table 1 below.

2. Multiple Site Modification

5   10 O.D. units (A_{260}) of Oligomer 13 (see Example 7) was
treated with cholic acid-NHS ester (Compound 1, 10 mg, 19.8
μmols) in 300 μl of 0.2 M $NaHCO_3$ buffer/ 50 μl DMF. The
reaction mixture was heated for 16 hrs at 45°C. The product
was isolated as per the method of Example 8-A-1. Analytical
HPLC revealed 58% doubly labeled product, 17% of a first
10   singly labeled product and 24% of a second singly labeled
product. Work up as per Example 8-A-1 gave the oligonucleo-
tide:

     Oligomer 24:   CTG TCT CCA* TCC TCT TCA* CT

wherein   A*   represents   nucleotides   functionalized   to
15   incorporate a cholic acid functionality linked via a 2'-O-
pentyl-amino linking group to the   2' position of the
designated nucleotide. HPLC retention times are shown in
Table 1 below.

D. Functionalization with Digoxigenin

1. Single Site Modification

20   10 O.D. units (A_{260}) of Oligomer 12 (see Example 7) was
treated with digoxigenin-3-O-methylcarbonyl-ϵ-aminocaproic N-
hydroxy succinimide ester (Boehringer Mannheim Corporation,
Indianapolis, IN) in 200 μl of 0.1 M borate pH 8.3 buffer/ 40
25   μl DMF. The reaction mixture was let stand overnight. The
product was isolated as per the method of Example 8-A-1. Work
up of the reaction gave the oligonucleotide:

     Oligomer 25:   CTG TCT CCA* TCC TCT TCA CT

wherein   A*   represents   a   nucleotide   functionalized   to
30   incorporate a digoxigenin functionality linked via a 2'-O-
pentyl-amino linking group to the   2' position of the
designated nucleotide. HPLC retention times are shown in
Table 1 below.

2. Multiple Site Modification

35   10 O.D. units (A_{260}) of Oligomer 13 (see Example 7) was
treated with digoxigenin-3-O-methylcarbonyl-ϵ-aminocaproic N-
hydroxy succinimide ester (Boehringer Mannheim Corporation,

2122030

30

Indianapolis, IN) in 300 μl of 0.1 M borate pH 8.3 buffer /
50 μl DMF. The reaction mixture was let stand overnight. The
product was isolated as per the method of Example 8-A-1. Work
up as per Example 8-A-1 gave the oligonucleotide:

5   Oligomer 26:   CTG TCT CCA* TCC TCT TCA* CT

wherein A* represents nucleotides functionalized to
incorporate a cholic acid functionality linked via a 2'-O-
pentyl-amino linking group to the 2' position of the
designated nucleotide. HPLC retention times are shown in
10 Table 1 below.

                          TABLE 1

          HPLC Retention Times Of Oligonucleotides Functionalized
                          At 2' Position

| | Retention Time Minutes | |
Oligomer	Mono Substitution	Multiple Substitution
Oligomer 12[1]	21.78	
Oligomer 13[1]		22.50
Oligomer 19[2]	23.58	
Oligomer 20[2]		24.16[a] 25.19[b]
Oligomer 21[3]	26.65	
Oligomer 22[3]		26.99[a] 29.33[b]
		27.55[a]
Oligomer 23[4]	30.10	
Oligomer 24[4]		30.38[a] 37.00[b]
		32.22[a]
Oligomer 25[5]	28.06	
Oligomer 26[5]		28.14[a] 33.32[b]
		29.24[a]

Conditions: Waters 600E with 991 detector, Hamilton PRP-1
column 0.7 x 15 cm; solvent A: 50 mM TEAA pH 7.0; B: 45 mM
TEAA with 80% acetonitrile: 1.5 ml flow rate: Gradient: 5%
B for first 5 mins., linear (1%) increase in B every minute
thereafter;
[a] Mono conjugated minor product;
[b] Doubly conjugated major product;
[1] Parent Oligonucleotide - no 2' functionalization;
[2] 2' Biotin functionalization;
[3] 2' Fluorescein functionalization;
[4] 2' Cholic Acid functionalization; and
[5] 2' Digoxigenin functionalization.

EXAMPLE 9

Functionalization Of Oligonucleotide At the 2' Position with Reporter Enzymes, Peptides and Proteins

      A.   Use of Heterobifunctional Linker

5   1.   Synthesis of Oligonucleotide-Maleimide Conjugate

Oligomer 12 (Example 7)(100 O.D. units, 600 nmols) is lyophilized in a 5 ml pear-shaped flask. Sulfo-SMCC reagent, Pierce Chemical Co. (Rockford, Il) (16 mg, 46 µmols) is

10   dissolved in phosphate buffer (800 µl, 0.1M, pH 7.0) and added to the oligonucleotide bearing flask. An additional 200 µl of buffer are used to wash the reagent and transfer it to the oligonucleotide flask. The contents of the flask are stirred overnight and loaded on to a Sephadex G-25 column (1 x 40 cm)

15   equipped with a fraction collector. The oligonucleotide-maleimide conjugate containing fractions are collected and tested by analytical HPLC for separation from other NHS type products.

        2. Synthesis of Oligonucleotide-Peptide Conjugate

20   An aliquot of the oligonucleotide-maleimide conjugate of Example 9-A-1 (about 50 O.D. units, 300 nmols) is lyophilized in a microfuge tube. SV40 peptide (pro-asp-lys-lys-arg-lys-cys)(2.5 mg, about 2.5 µmols) is taken up in phosphate buffer (800 µl, 0.1 M, pH 7.0) and added to the

25   oligonucleotide-maleimide conjugate containing tube. The contents of the tube are stirred overnight under an argon atmosphere. The reaction mixture is passed through a Sephadex G-25 column and the oligonucleotide-peptide conjugate fractions are identified by HPLC. Isolation of the product

30   from product-bearing fractions via HPLC and desalting on Sephadex G-25 will yield an oligonucleotide of the sequence:

       Oligomer 27:   CTG TCT CCA* TCC TCT TCA CT

wherein A* represents a nucleotide functionalized to incorporate a SV40 peptide functionality linked via a 2'-O-

35   pentyl-amino-sulfo-SMCC   (sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) linking group to the 2' position of the designated nucleotide.

2122030   32

B. Use of Homobifunctional Linker

1. Synthesis of Oligonucleotide-Disuccinimidyl Suberate Conjugate

An aliquot (10 O.D. units, 60 nmols) of Oligomer 12
5   (Example 7) is evaporated to dryness and is dissolved in
freshly prepared 0.1 M NaHCO$_3$/50 mM EDTA (100 µl, pH 8.25).
The solution is then treated with a solution of DSS, Pierce
Chemical Co. (Rockford, Il) (2.6 mg, 7 µmol) in 200 µl DMSO.
The solution is stored at room temperature for 15 minutes and
10   then immediately applied to a Sephadex G-25 column (1 x 40 cm)
that is previously packed and washed with water at 4°C. The
oligonucleotide fractions are combined immediately in a 25 ml
pear-shaped flask and are rapidly frozen in dry ice/isopropyl
alcohol and lyophilized to a powder.

15   **2. Synthesis of Oligonucleotide-Protein Conjugate**

A solution of calf intestinal alkaline phosphatase
(Boehringer Mannheim) (20.6 mg, 2.06 ml, 147 nmol) is spun at
4°C in a Centricon microconcentrator at 6000 rpm until the
volume is less than 50 µl. It is then redissolved in 1 ml of
20   cold Tris buffer (pH 8.5, 0.1M containing 0.1 NaCl and 0.05
M MgCl$_2$) and concentrated twice more. Finally the concentrate
is dissolved in 400 µl of the same buffer. This solution is
added to the activated oligonucleotide from Example 9-B-1 and
the solution stored for 18 hrs at room temp. The product is
25   diluted to approximately 30 ml and applied to a Sephadex G-25
column (1 x 20 cm, chloride form) maintained at 4°C. The
column is eluted with 50 nM Tris-Cl pH 8.5 until the UV
absorbance of the fractions eluted reach near zero values.
The column is then eluted with a NaCl salt gradient 0.05 M to
30   0.75 M (150 ml each). The different peaks are assayed for
both oligonucleotide and alkaline phosphatase activity and the
product bearing fractions are combined. Typically the first
peak will be excess enzyme, the second peak the
oligonucleotide-protein conjugate and the third peak unreacted
35   oligonucleotide. Isolation of the product from the product-
bearing fractions via HPLC and desalting on Sephadex G-25
will yield an oligonucleotide of the sequence:

Oligomer 28:　　CTG TCT CCA TCC TCT TCA CT

wherein A* represents a nucleotide functionalized to incorporate an alkaline phosphatase functionality linked via an 2'-O-pentyl-amino-sulfo-SMCC (sulfosuccinimidyl-4-(N-

5 maleimidomethyl)cyclohexane-1-carboxylate) linking group to the 2' position of the designated nucleotide.

EXAMPLE 10

Heterocyclic Base Peptide-Conjugated Oligonucleotides

Utilizing the method of Example 9-A-1 the intermediate

10 amino linker oligonucleotide of Example 2-A is reacted with sulfo-SMCC reagent. The isolated oligonucleotide-maleimide conjugate is then further reacted with SV40 peptide as per Example 9-A-2. This will give an oligonucleotide of the structure:

15　　　　Oligomer 29:　　TTG CTT* CCA TCT TCC TCG TC

wherein T* represents a nucleotide functionalized to include a peptide linked via an extended linker to the heterocyclic base of a 2'-deoxyuridine nucleotide.

EXAMPLE 11

20 ## 3'-Terminus Protein-Conjugated Oligonucleotides

Utilizing the method of Example 9-B-1 the 2'-O-methyl derivatized intermediate amino linker oligonucleotide of Example 6-A is reacted with DSS reagent. The isolated oligonucleotide-disuccinimidyl suberate conjugate is then

25 further reacted with a lysine containing Nuclease RNase H using the method of Example 9-B-2. This will give an oligonucleotide of the structure:

Oligomer 30:　　$C_sC_sC_s$ $A_sG_sG_s$ $C_sU_sC_s$ A_sG_sA-3'-protein

wherein protein represents RNase H, the subscript "s"

30 represents a phosphorothioate inter-nucleotide backbone linkage and each of the nucleotides of the oligonucleotide includes a 2'-O-methyl group thereon.

EXAMPLE 12

5'-Terminus   Protein-Conjugated   2'-O-Methyl   Derivatized Oligonucleotides

Utilizing the method of Example 9-B-1 the 2'-O-methyl
5 derivatized intermediate amino linker oligonucleotide of
Example 6-A (Oligomer 6) is reacted with DSS reagent. The
isolated oligonucleotide-disuccinimidyl suberate conjugate is
then further reacted with a lysine containing Staphylococcal
Nuclease using the method of Example 9-B-2. This will give
10 an oligonucleotide of the structure:

Oligomer 31:   5'-protein-C$_s$C$_s$C$_s$ A$_s$G$_s$G$_s$ C$_s$U$_s$C$_s$ A$_s$G$_s$A 3'
wherein protein represents Staphylococcal Nuclease, the
subscript "s" represents a phosphorothioate inter-nucleotide
backbone linkage and each of the nucleotides of the
15 oligonucleotide includes a 2'-O-methyl group thereon.

PROCEDURE A

Confirmation of Structure of Functionalized Oligonucleotides Containing A Tethered 2'-Amino Moiety

Oligonucleotides of the invention were digested with
20 snake venom phosphodiesterase and calf-intestine alkaline
phosphatase to their individual nucleosides. After digestion,
the nucleoside composition was analyzed by HPLC. The HPLC
analysis established that functionalized nucleotide compounds
having the tethered 2'-amino moiety thereon were correctly
25 incorporated into the oligonucleotide.

Snake venom phosphodiesterase [Boehringer-Mannheim cat.
#108260, 1 mg (1.5 units)/0.5 ml] and alkaline phosphatase
from calf intestine (1 unit/microliter, Boehringer-Mannheim
cat. # 713023) in Tris-HCl buffer (pH 7.2, 50 mM) were used
30 to digest the oligonucleotides to their component nucleosides.
To 0.5 O.D. units of oligonucleotide in 50 μl buffer (nearly
40 μM final concentration for a 20 mer) was added 5 μl of
snake venom phosphodiesterase (nearly 0.3 units/mL, final
concentration) and 10 μl of alkaline phosphatase (app. 150
35 units/mL, final concentration). The reaction mixture was
incubated at 37°C for 3 hours. Following incubation, the
reaction mixture was analyzed by HPLC using a reverse phase

2122030

analytical column (app. 30 x 2.5 cm); solvent A: 50 mM TEAA
pH 7; solvent B: acetonitrile; gradient 100% for 10 mins, then
5% B for 15 mins, then 10% B and then wash. The results of
these digestion are shown in Table 2 for representative
5 oligonucleotides.

TABLE 2
OLIGONUCLEOTIDE ANALYSIS VIA ENZYMATIC DIGESTION

Oligomer	Abs. max.	Observed Ratios[**]				
		267 C	252 G	267 T	260 A	A
Oligomer 10		2	1		1	
Oligomer 11		3	5	2	1	
Oligomer 12		9	1	8	1	1
Oligomer 13		9	1	8	2	

[*] Nucleoside having 2'-O-linker attached thereto; and
[**] Corrected to whole numbers.

As is evident from Table 2, the correct nucleoside ratios are
20 observed   for   the   component   nucleotides   of   the   test
oligonucleotides.

PROCEDURE B

**Determination of Melting Temperatures (Tm's) of Cholic Acid
Oligonucleotide Conjugates**

25   The relative ability of oligonucleotides to bind to
their complementary strand is compared by determining the
melting temperature of the hybridization complex of the
oligonucleotide and its complementary strand.   The melting
temperature (Tm), a characteristic physical property of double
30 helices, denotes the temperature in degrees centigrade at
which 50% helical versus coil (un-hybridized) forms are
present. Tm is measured by using the UV spectrum to determine
the formation and breakdown (melting) of hybridization. Base
stacking, which occurs during hybridization, is accompanied
35 by   a   reduction   in   UV   absorption   (hypochromicity).
Consequently a reduction in UV absorption indicates a higher
T_m.   The higher the Tm, the greater the strength of the
binding of the strands. Non-Watson-Crick base pairing has a
strong destabilizing effect on the Tm. Consequently, absolute

fidelity of base pairing is necessary to have optimal binding
of an antisense oligonucleotide to its targeted RNA.

1. Terminal End Conjugates
a. Synthesis

5   A series of oligonucleotides were synthesized utilizing
standard synthetic procedures (for un-functionalized
oligonucleotides) or the procedure of Example 3-A above for
oligonucleotides having a 5'-terminus amino linker bearing
oligonucleotide or the procedure of Example 3-B for 5'-
10 terminus cholic acid-bearing oligonucleotides. Each of the
oligonucleotides had the following 5-LO antisense sequence:
5' TCC AGG TGT CCG CAT C 3'. The nucleotides were synthesized
on a 1.0 μmol scale. Oligomer 32 was the parent compound
having normal phosphodiester inter-nucleotide linkages.
15 Oligomer 33 incorporated phosphorothioate inter-nucleotide
linkages in the basic oligonucleotide sequence. Oligomer 34
is a an intermediate oligonucleotide having a 5'-aminolink at
the 5'-terminus of the basic oligonucleotide sequence and
Oligomer 35 was a similar 5'-aminolink compound incorporating
20 phosphorothioate inter-nucleotide linkages. Oligomer 36 is
a 5'-terminus cholic acid conjugate of the basic
phosphodiester oligonucleotide sequence while Oligomer 37 is
a similar 5'-cholic acid conjugate incorporating
phosphorothioate inter-nucleotide linkages. Oligomers 32 and
25 33 were synthesized in a "Trityl-On" mode and were purified
by HPLC. Oligomers 34 and 35 were synthesized as per Example
30-A above without or with Beaucage reagent treatment, to
yield phosphodiester or phosphorothioate inter-nucleotide
linkages, respectively. Oligomers 36 and 37 were prepared
30 from samples of Oligomers 34 and 35, respectively, utilizing
a solution of cholic acid N-hydroxysuccinimide ester (Compound
1) 1 dissolved in DMF as per Example 3-B. Oligomers 36 and
37 were purified by HPLC. The products were concentrated and
desalted in a Sephadex G-25 column. Gel electrophoresis
35 analyses also confirmed a pure product with the pure conjugate
moving slower than the parent oligonucleotide or 5'-amino
functionalized oligonucleotide.

b. Melting Analysis

The test oligonucleotides [either the phosphodiester, phosphorothioate, cholic acid conjugated phosphodiester, cholic acid conjugated phosphorothioate or 5'-aminolink
5 intermediate phosphodiester or phosphorothioate oligonucleotides of the invention or otherwise] and either the complementary DNA or RNA oligonucleotides were incubated at a standard concentration of 4 uM for each oligonucleotide in buffer (100 mM NaCl, 10 mM Na-phosphate, pH 7.0, 0.1 mM EDTA).
10 Samples were heated to 90 degrees C and the initial absorbance taken using a Guilford Response II spectrophotometer (Corning). Samples were then slowly cooled to 15 degrees C and then the change in absorbance at 260 nm was monitored during the heat denaturation procedure. The temperature was
15 elevated 1 degree/absorbance reading and the denaturation profile analyzed by taking the 1st derivative of the melting curve. Data was also analyzed using a two-state linear regression analysis to determine the Tm's. The results of these tests are shown in Table 3 as are the HPLC retention
20 times of certain of the test compounds.

TABLE 3

Melting Temperature Of The Hybridization Complex Of The
Oligonucleotide And Its Complementary Strand

| Oligomer | Tm** | | HPLC Ret. Time* |
	DNA	RNA	minutes
32	62.6	62.0	----
33	55.4	54.9	----
34	ND	ND	13.6
35	ND	ND	17.0
36	63.4	62.4	22.0
37	56.3	55.8	22.5

*   HPLC conditions: Walters Delta Pak C-18 RP 2.5u column; at 0 min 100% 0.1 TEAA; at 30 min 50% TEAA and 50% Acetonitrile: Flow rate 1.0 ml/min.

**   Tm at 4μM each strand from fit of duplicate melting curves to 2-state model with linear

sloping base line. Conditions: 100 mM NaCl, 10 mM
Phosphate, 0.1 mM EDTA, pH 7.0.

ND = not determined

As is evident from Table 2, conjugates of cholic acid
5 at the end of the oligonucleotide do not affect the Tm of the
oligonucleotides.

   2. Strands Incorporating 2'-O-Pentylamino Linker
       a. Synthesis
   An oligonucleotide of the sequence:
10   Oligomer 38:   GGA* CCG GA*A* GGT A*CG A*G
wherein A* represents a nucleotide functionalized to
incorporate a pentylamino functionality at its 2'-position was
synthesized in a one micromole scale utilizing the method of
Example 7-B.   The oligonucleotide was purified by reverse
15 phase HPLC, detritylated and desalted on Sephadex G-25. PAGE
gel analysis showed a single band. A further oligonucleotide,
Oligomer 39, having the same sequence but without any 2'-O-
amino linker was synthesis in a standard manner.   A
complementary DNA oligonucleotide of the sequence:
20   Oligomer 40:   CCT GGC CTT CCA TGC TC.
was also synthesized in a standard manner as was a
complementary RNA oligonucleotide of the sequence:
       Oligomer 41:   CCU GGC CUU CCA UGC UC
       b. Melting Analysis
25   Melting analysis was conducted as per the method of
Procedure B-1-b. The results are shown in Table 4.

TABLE 4

Melting Temperature Of The Hybridization Complex Of
The Oligonucleotide And Its Complementary Strand

| Oligomer | Tm^* | |
	DNA^1	RNA^2
38	54.5	58.0
39	60.6	56.9

* Tm at $4\mu M$ each strand from fit of duplicate
melting curves to 2-state model with linear
sloping base line. Conditions: 100 mM NaCl, 10 mM
Phosphate, 0.1 mM EDTA, pH 7.0.
1 Against DNA complementary strand, Oligomer 40.
2 Against RNA complementary strand, Oligomer 41

As is evident from Table 4 against the RNA
complementary strand the change in Tm's between the strand
having 2'-amino linkers thereon and the unmodified strand is
1.1 degrees (0.22 change per modification). Against the DNA
strand, the change is -6.1 degrees (-1.2 change per modifica-
tion). When compared to the parent unmodified oligonucleotide
the 2'-amino linker- containing strand has a stabilizing
effect upon hybridization with RNA and a destabilizing effect
upon hybridization with DNA.

Compounds of the invention were tested for their
ability to increase cellular uptake. This was determined by
judging either their ability to inhibit the expression of
bovine papilloma virus-1 (BPV-1) or an assay involving
luciferase production (for HIV-1).

PROCEDURE C

Determination of Cellular Uptake Judged By The Inhibition Of
Expression of Bovine Papilloma Virus-1 (bpv-1) As Measured By
an E2 Transactivation Assay

For this test, a phosphorothioate oligonucleotide
analog of the sequence:

Oligomer 42: CTG TCT CCA TCC TCT TCA CT

was used as the basic sequence. This sequence is designed to
be complementary to the translation initiation region of the
E2 gene of bovine papilloma virus type 1 (BPV-1). Oligomer
42 served as the positive control and standard for the assay.

Oligomer 3 (from Example 4 above) served as a second test compound. It has the same basic sequence except it is a phosphorothioate oligonucleotide and further it has a cholic acid moiety conjugated at the 3'-end of the oligonucleotide.

5   Oligomer 2 (from Example 2 above) served as a third test compound. Again it is of the same sequence, it is a phosphorothioate oligonucleotide and it has a cholic acid moiety conjugated at the 5'-end. Oligomer 5 (from Example 5 above) served as a fourth test compound. Once again it has

10   the same sequence, is a phosphorothioate oligonucleotide and it has a cholic acid moiety conjugated at both the 3'-end and 5'-end. A fifth test compound was a phosphorothioate oligonucleotide with no significant sequence homology with BPV-1. A sixth test compound was a further phosphorothioate

15   oligonucleotide with no significant sequence homology with BPV-1. The last test compound, the seventh test compound, was a phosphorothioate oligonucleotide with cholic acid conjugated to the 3'-end but having no significant sequence homology with BPV-1. Compounds five, six and seven served as negative

20   controls for the assay.

For each test I-38 cells were plated at 5×10^4 cells per cm^2 in 60 mm petri dishes. Eight hours after plating, medium was aspirated and replaced with medium containing the test oligonucleotide and incubated overnight. Following

25   incubation, medium was aspirated and replaced with fresh medium without oligonucleotide and incubated for one hour. Cells were then transfected by the $CaPO_4$ method with 2 ug of pE2RE-1-CAT. After a four hour incubation period cells were glycerol shocked (15% glycerol) for 1 minute followed by

30   washing 2 times with PBS. Medium was replaced with DMEM containing oligonucleotide at the original concentration. Cells were incubated for 48 hours and harvested. Cell lysates were analyzed for chloramphenicol acetyl transferase by standard procedures. Acetylated and nonacetylated ^{14}C-

35   chloramphenicol were separated by thin layer chromatography and quantitated by liquid scintillation. The results are expressed as percent acetylation.

Two lots of the positive control compound were found
to acetylate at a level of 29% and 30%. The negative
controls, test compounds five, six and seven, were found to
acetylate at 59%, 58% and 47%, respectively. The 3'-cholic
5 acid conjugate test compound, Oligomer 3, was found to
acetylate to 23%, the 5'-cholic acid conjugate test compound,
Oligomer 2, was found to acetylate to 36% and the test
compound conjugated at both the 3'-end and the 5'-end,
Oligomer 5, was found to acetylate to 27%.

10   The results of this test suggests that placement of a
cholic acid moiety at the 3'-terminus of an oligonucleotide
increase the activity. This in turn suggests that the
increased activity was the result of increased cellular
membrane transport.

15 **PROCEDURE D**

**Determination of Cellular Uptake Judged By Inhibition of
pHIVluc With Cholic Acid Linked 2'-O-Methyl Substituted
Oligonucleotides**

For this test the absence of an oligonucleotide in a
20 test well served as the control. All oligonucleotides were
tested as 2'-O-methyl analogs. For this test an oligonucleo-
tide of the sequence:

      Oligomer 43:   CCC AGG CUC AGA

where each of the nucleotides of the oligonucleotide includes
25 a 2'-O-methyl substituent group served as the basic test
compound. The second test compound of the sequence:

      Oligomer 44:   5'-CHA CCC AGG CUC AGA

wherein CHA represents cholic acid and where each of the
nucleotides of the oligonucleotide includes a 2'-O-methyl
30 substituent group, was also of the same sequence as the first
test compound. This second test compound included cholic acid
conjugated to its 5'-end and was prepared as per the method
of Example 3 utilizing 2'-O-methyl phosphoramidite
intermediates as identified in Example 7-C. The third test
35 compound of the sequence:

      Oligomer 45:   CCC AGG CUC AGA 3'-CHA

wherein CHA represents cholic acid and where each of the
nucleotides of the oligonucleotide includes a 2'-O-methyl
substituent group was also of the same sequence as the first
test compound. The third test compound included cholic acid
5   conjugated to its 3'-end and was prepared as per the method
of Example 4 utilizing 2'-O-methyl phosphoramidite
intermediates as identified in Example 7-C. The fourth test
compound was a 2'-O-Me oligonucleotide of a second sequence:
        Oligomer 46:   GAG CUC CCA GGC
10 where each of the nucleotides of the oligonucleotide includes
a 2'-O-methyl substituent group. The fifth test compound was
of sequence:
        Oligomer 47:   5'-CHA GAG CUC CCA GGC.
wherein CHA represents cholic acid and where each of the
15 nucleotides of the oligonucleotide includes a 2'-O-methyl
substituent group. It was of the same sequence as the fifth
test compound. This test compound included cholic acid
conjugated to its 5'-end and was prepared as per the method
of Example 3 utilizing 2'-O-methyl phosphoramidite
20 intermediates as identified in Example 7-C.
A sixth test compound was a randomized oligonucleotide of the
sequence:
        Oligomer 48:   CAU GCU GCA GCC.
        HeLa cells were seeded at 4×10^5 cells per well in 6-
25 well culture dishes. Test oligonucleotides were added to
triplicate wells at 1 μM and allowed to incubate at 37°C for
20 hours. Medium and oligonucleotide were then removed, cells
washed with PBS and the cells were CaPO$_4$ transfected.
Briefly, 5μg of pHIVluc, a plasmid expressing the luciferase
30 cDNA under the transcriptional control of the HIV LTR
constructed by ligating the KpnI/HindIII restriction fragments
of the plasmids pT3/T7luc and pHIVpap (NAR 19(12)) containing
the luciferase cDNA and the HIV LTR respectively, and 6 μg of
pcDEBtat, a plasmid expressing the HIV tat protein under the
35 control of the SV40 promoter, were added to 500 μl of 250 mM
CaCl$_2$, then 500 μl of 2x HBS was added followed by vortexing.
After 30 minutes, the CaPO$_4$ precipitate was divided evenly

43

between the six wells of the plate, which was then incubated
for 4 hours. The media and precipitate were then removed, the
cells washed with PBS, and fresh oligonucleotide and media
were added. Incubation was continued overnight. Luciferase
5 activity was determined for each well the following morning.
Media was removed, then the cells washed 2X with PBS. The
cells were then lysed on the plate with 200 μl of LB (1% Trit
X-100, 25 mM Glycylglycine pH 7.8, 15 mM MgSO$_4$, 4 mM EGTA, 1mM
DTT). A 75 μl aliquot from each well was then added to a well
10 of a 96 well plate along with 75 μl of assay buffer (25 mM
Glycylglycine pH 7.8, 15 mM MgSO$_4$, 4 mM EGTA, 15 mM KPO$_4$, 1 mM
DTT, 2.5 mM ATP). The plate was then read in a Dynatec
multiwell luminometer that injected 75 μl of Luciferin buffer
(25 mM Glycylglycine pH 7.8, 15 mM MgSO$_4$, 4 mM EGTA, 4 mM DTT,
15 1 mM luciferin) into each well, immediately reading the light
emitted (light units).

The random sequence compound (Oligomer 48) and the
other non-cholic acid-conjugated test compounds (Oligomers 43
and 46) had comparable activity. The 5'-conjugate of the
20 first sequence (Oligomer 44) also had activity comparable to
the non-conjugated compounds. The 5'-conjugate of the second
sequence (Oligomer 47) showed a three-fold increase in
activity compared to the non-conjugated compounds and the 3'-
conjugate of the first sequence (Oligomer 45) showed a further
25 3-fold increase in activity compared to Oligomer 47.

All the test cholic acid-bearing oligonucleotides
showed significant inhibition of luciferase production
compared to non-cholic acid- bearing oligonucleotides. This
suggests that the increased activity was the result of
30 increased cellular membrane transport of the cholic acid-
bearing test oligonucleotides.

EXAMPLE 13
Retinoic Acid Conjugated Oligonucleotide
      A. Retinoic Acid N-Hydroxysuccinimide Ester
35   Anhydrous DMF (150 ml) was added to a mixture of
retinoic acid (15 mmol, 4.5 g, Fluka) and N-hydroxysuccinimide

(5.25 g, 45 mmol). The mixture was stirred in the presence
of argon. EDAC [ethyl-3-(3-dimethylamino)propyl carbodiimide]
(4 ml, 25 nmol) was then added and this mixture was then
stirred overnight. The solution was then evaporated to a
5 yellow gum and dissolved in 200 ml ethylacetate and washed
successively with 4% NaHCO$_3$ solution (200 ml) followed by
saturated NaCl solution, dried over anhydrous MgSO$_4$ and
evaporated to yield the desired compound as a yellow solid in
nearly 90% yield.

10   B. Retinol Phosphoramidite
           All-_trans_-retinol (1 g) was vacuum dried and dissolved
in anhydrous CH$_2$Cl$_2$ (10 ml) in an argon atmosphere.
Diisopropylethylamine (2.65 ml, 21.5 mmol) was syringed in and
the reaction mixture was cooled in an ice-bath. 2-cyanoethyl-
15 N, N-diisopropylchlorophosphoramidate (2.5 g, 2.45 ml, 10.5
mmol) was slowly added by syringe under argon atmosphere. The
reaction mixture was stirred for 30 min. at which time TLC
(CH$_2$Cl$_2$:CH$_3$OH:Et$_3$N, 90:10:0.1) indicated complete conversion of
the alcohol to its phosphoramidite. The reaction mixture was
20 added to 100 ml of saturated NaHCO$_3$ followed by washing the
reaction flask with (2 x 25 ml) CH$_2$Cl$_2$. The CH$_2$Cl$_2$ layer was
separated and washed with 100 ml of saturated NaCl solution
and dried over anhydrous MgSO$_4$ and evaporated into a yellow
foam. The amidite was used directly in the DNA synthesizer
25 without further purification since decomposition of this
amidite was noted upon silica column purification.

          C. Retinoic Acid Functionalized Oligonucleotide
           Multiple batches of an oligonucleotide of the sequence:
           Oligomer 49:
30   T$_s$G$_s$G$_s$ G$_s$A$_s$G$_s$ C$_s$C$_s$G$_s$ T$_s$A$_s$G$_s$ C$_s$G$_s$A$_s$ G$_s$G$_s$C$_s$-3'AL
wherein AL represents a 3'-aminolinker and "s" represents a
phosphorothioate inter-nucleotide backbone linkage were
synthesized as per the procedure of Example 4 on 10 μmol
scales in the standard manner on the DNA synthesizer utilizing
35 phosphoramidite methodology employing 3'-amine-ON solid
support available from Clontech. During the synthesis, the
phosphorothioate backbone was formed by the Beaucage reagent.

The oligonucleotide was deprotected and purified using
standard protocols.

The 3'-aminolinker-oligonucleotide (Oligomer 49, 100
OD units, approximately 550 nmols) was dissolved in freshly
5   prepared NaHCO3 buffer (500 μl, 0.2M, pH 8.1) and treated with
a solution of retinoic acid N-hydroxy succinimide ester (50
mg, 125 μmols) dissolved in 500 μl of DMF. The reaction
mixture was covered with aluminum foil and left at 37°C bath
overnight. It was then passed through a Sephadex G-25 column
10 (1 x 40 cm) and the first eluant was collected, concentrated
and passed again through another Sephadex G-25 column (1 x 40
cm) to remove excess Vitamin-A reagent. Concentration of the
yellow oligonucleotide fractions followed by HPLC purification
yielded the retinoic acid-oligonucleotide conjugate.

15 **EXAMPLE 14**
    Folic Acid Conjugated Oligonucleotide
A mixture of folic acid (30 mg, 68 μmols) and 1-
hydroxybenzotriazole (30 mg, 222 μmols) was dissolved in 900
ml of dry DMF. To this solution, 50 ml of EDAC (312 μmols)
20 was added. The resultant yellow viscous material was vortexed
well and 500 ml from the solution was transferred into 100
O.D. units of the 3'-aminolinker oligonucleotide (Oligomer 49,
537 μmols) dissolved in 0.2M NaHCO$_3$ buffer. The yellow
solution was vortexed, covered with aluminum foil and allowed
25 to react for 16 hrs. The mixture was then loaded into a
Sephadex G-25 column (1 x 40 cm). The oligonucleotide
fraction was collected, concentrated and passed one more time
through Sephadex G-25 column. The oligonucleotide fractions
were concentrated and purified by reverse phase HPLC. The
30 conjugate appeared as a broad peak centered around 32.5 min.
while the oligonucleotide starting material had a retention
time of 30 min. (5% → 40% of 100% CH$_3$CN over 60 min. as
solvent B and 50 mM TEAA pH 7.0 as the solvent A in reverse
phase HPLC). The conjugate was concentrated and passed
35 through Sephadex G-25 column again for desalting. Gel

analysis indicated a slower moving material than the starting
oligonucleotide.

EXAMPLE 15
Methyl Folate Conjugated Oligonucleotide

5   In a like manner to Example 14, 5-methyl folate was
also attached to Oligomer 49.

EXAMPLE 16
Pyridoxal Conjugated Oligonucleotide

          The 3'-aminolinker-oligonucleotide (Oligomer 49, 20
10 O.D. units, approximately 110 nmols, based on the calculated
extinction coefficient of 1.828×10^5 at 260 nm) was dissolved
in 100 microliters of water. 100 ml of 1M NaOAc buffer (pH
5.0) was added followed by 5 mg of pyridoxal hydrochloride (24
μmols) and 50 μl of 60 mM NaCNBH$_3$ solution. The solution was
15 vortexed and left aside for overnight. It was then passed
through a Sephadex G-25 column and further purified in an
analytical HPLC column.

EXAMPLE 17
Tocopherol Conjugated Oligonucleotide

20   A.   Vitamin E (Tocopherol)-hemisuccinate-NHS ester
          α-Tocopherolhemisuccinate (Sigma, 5 g, 9.4 mmols) was
treated with 3 equivalents of N-hydroxysuccinimide and 2
equivalents of EDAC as described under the vitamin-A NHS ester
synthesis, Example 13-A above. Work up in the same manner as
25 Example 13 yielded the title compound as a light brown wax-
like solid.   .   .   . -   .   - ..
          B.   Tocopherol Conjugated Oligonucleotide
          α-Tocopherol-hemisuccinate-NHS ester was treated with
Oligomer 49 in the same manner described in Example 13-C for
30 the retinoic acid conjugation. The conjugate was obtained in
nearly 50% yield.   . .

EXAMPLE 18

Synthesis of Uridine Based Aminolinkers

A.   Preparation of 5'-dimethoxytrityl-2'-(O-pentyl-N-phthalimido)uridine phosphoramidite

5   Utilizing the protocol of Wagner, et al., J. Org. Chem. 1974, 39, 24, uridine (45 g, 0.184 mol) was refluxed with di-n-butyltinoxide (45 g, 0.181 mol) in 1.4 l of anhydrous methanol for 4 hrs. The solvent was filtered and the resultant 2',3'-O-dibutylstannylene-uridine was dried under

10   vacuum at 100°C for 4 hrs to yield 81 g (93%).

       The 2',3'-O-dibutyl stannylene-uridine was dried over P_2O_5 under vacuum for 12 hrs. To a solution of this compound (20 g, 42.1 mmols) in 500 ml of anhydrous DMF were added 25 g (84.2 nmols) of N(5-bromopentyl)phthalimide (Trans World

15   Chemicals, Rockville, Maryland) and 12.75 g (85 mmols) of cesium fluoride (CeF) and the mixture was stirred at room temperature for 72 hrs. The reaction mixture evaporated, coevaporated once with toluene and the white residue was partitioned between EtOAc and water (400 ml each). The EtOAC

20   layer was concentrated and applied to a silica column (700 g). Elution with CH_2Cl_2-CH_3OH (20:1 v/v) gave fractions containing a mixture of the 2'- and 3'- isomers of O-pentyl-ω-N-phthalimido uridine, in 50% yield.

       The mixture was allowed to react with DMT chloride in

25   dry pyridine at room temperature for 6 hrs. CH_3OH was used to quench excess DMT-Cl and the residue was partitioned between CH_2Cl_2 containing 0.5% Et_3N and water. The organic layer was dried ($MgSO_4$) and the residue was applied to a silica column. Elution with CH_2Cl_2:CH_3OH (20:1, v/v) separated the 2' and 3'

30   isomers.

       The 2'-O-pentyl-ω-N-phthalimido-5'-DMT-uridine was converted to its phosphoramidite as per the procedure referenced in Example 7.

B.   Preparation of 5'-dimethoxytrityl-2-(O-hexyl-N-
phthalimido)uridine phosphoramidite

In a like manner to Example 18-A, using N-(6-
bromohexyl) phthalimide, a 2'- six carbon aminolinker was
5   introduced at the 2'-position of uridine.

C.   Preparation of 5'-dimethoxytrityl-2-(O-decyl-N-
phthalimido)uridine phosphoramidite

In a like manner to Example 18-A N-(10-
bromodecyl)phthalimide was similarly used to introduce a 2'-
10   ten carbon aminolinker in the nucleotide.

EXAMPLE 19
Synthesis of Cytidine Based Aminolinkers

A.   Preparation of 5'-dimethoxytrityl-2-(O-propyl-N-
phthalimido)cytidine phosphoramidite

15   The 5'-DMT protected 2'-O-functionalized cytidine
phosphoramidite was prepared as per the procedure of Example
7 substituting cytidine for adenosine.

B.   Preparation of oligonucleotides having a 2'-
aminolinker bearing 3'-terminal nucleotide

20   The following oligonucleotides having phosphodiester
inter-nucleotide linkages and a 2'-aminolinker at the 3'
terminal nucleotide were synthesized:

Oligomer 50:   GGC GUC UCC AGG GGA UCU GAC*
Oligomer 51:   TCT GAG TAG CAG AGG AGC TC*

25   wherein C* represents a nucleotide functionalized to
incorporate a propyl-N-phthalimido functionality. Oligomer
50 is antisense to the Cap region of CMV and Oligomer 51 is
antisense to an ICAM sequence. The oligonucleotides were
synthesized on a 3 μmol scale. Upon completion of synthesis
30   they were deprotected using standard protocols and purified
by reverse phase HPLC, detritylated and desalted.

EXAMPLE 20

Conversion Of An Oligonucleotide Having A 2'-Aminolinker To An Oligonucleotide Having A Thiolinker

      A.   Oligomer 50

5   Oligomer 50 (25 O.D. units) was treated with 5 mg SATA (N-succinimidyl-S-acetylthioacetate) in 0.2M NaHCO$_3$ buffer. The reaction mixture was passed through a Sephadex G-25 column, the oligonucleotide fraction was concentrated and treated with 200 mM NH$_2$OH hydrochloride solution in water (1

10 ml).

      B.   Oligomer 51

      Oligomer 51 (25 O.D. units) was treated with 5 mg SATA (N-succinimidyl-S-acetylthioacetate) in 0.2M NaHCO$_3$ buffer. The reaction mixture was passed through a Sephadex G-25

15 column, the oligonucleotide fraction was concentrated and treated with 200 mM hydroxylamine hydrochloride solution in water (1 ml).

EXAMPLE 21

Conjugation   of   *o*-Phenanthroline   at   2'-Position   Of

20 **Oligonucleotides**

      A.   Oligomer 52

      To the solution resulting from Example 20-A was added 2 mg of 5-(iodoacetamide)-*o*-phenanthroline reagent followed by shaking overnight. The conjugate was purified by a size

25 exclusion column and reverse phase HPLC to yield

      Oligomer 52:   GGC GUC UCC AGG GGA UCU GAC-2'PHA

wherein PHA represents a nucleotide functionalized at its 2'-position with phenanthroline via a thiol linker of the structure 2'-O-(CH$_2$)$_3$-NH-C(=O)-CH$_2$-S-CH$_2$-C(=O)-NH-.

30   **B.   Oligomer 53**

      To the solution resulting from Example 20-A was added 2 mg of 5-(iodoacetamide)-O-phenanthroline reagent followed by shaking overnight. The conjugate was purified by a size exclusion column and reverse phase HPLC to yield

35   Oligomer 53:   TCT GAG TAG CAG AGG AGC TC-2'PHA

wherein PHA represents a nucleotide functionalized at its 2'-position with phenanthroline via a thiol linker of the structure 2'-O-$(CH_2)_3$-NH-C(=O)-CH_2-S-CH_2-C(=O)-NH-.

EXAMPLE 22

5 **Oligonucleotide Having A Nucleotide With A Crosslinker/Alkylator Attached Via A 2'-Aminolinker In A Internal Position In The Oligonucleotide**

A. Synthesis of an oligonucleotide having a uridine 2'-aminolinker

10   The following oligonucleotide having phosphodiester inter-nucleotide linkages and a 2'-aminolinker at an internal position is synthesized utilizing the uridine 2'-aminolinker of Example 18:

        Oligomer 54:   GGC CAG AUC UGA GCC UGG GAG CU*C UGU GGC

15 C

wherein U* represents a nucleotide functionalized to incorporate a propyl-N-phthalimido functionality. Oligomer 50 is an oligonucleotide corresponding to positions G_{16} to C_{46} of TAR RNA.

20   **B. Conjugation of iodo acetamide to U_{38} position of TAR structure**

        Oligomer 54 is reacted with iodoacetic acid N-hydroxy-succinimide ester to form the iodoacetamide derivative at the U_{38} position of the TAR structure. The U_{38} position is thus

25 available for crosslinking to the 7 position of the guanine base of either G_{26} or G_{28} of the TAR structure.

EXAMPLE 23

Conjugation of Pyrene at 2'-Position Of Oligonucleotides

        A. Single 2' site modification

30   10 O.D. units (A_{260}) of Oligomer 12 (Example 7-B) (approximately 60 nmols based on the calculated extinction coefficient of 1.68×10^5) was dried in a microfuge tube. It was dissolved in 200 μml of 0.2 M $NaHCO_3$ buffer and pyrene-1-butyric acid N-hydroxysuccinimide ester (i.e., succinimidyl-1-

pyrene butyrate, 3 mg, 7.79 μmols, Molecular Probes, Eugene, Oregon) was added followed by 400 μl of DMF. The mixture was incubated at 37°C overnight. The solution was applied to a Sephadex G-25 column (1 x 40 cm) and the oligonucleotide
5   fractions were combined. The product was purified by HPLC. The pyrene conjugates exhibited the typical pyrene absorption between 300 and 400 nm. The product had a HPLC retention time of 26.94 min. while the parent oligonucleotide had a retention time of 21.78 min. (Waters 600E with 991 detector; Hamilton
10   PRP-1 column (15x25 cm); Solvent A: 50 mM TEAA, pH 7.0; B: 45 mM TEAA with 80% Acetonitrile; 1.5 mL/min. flow rate: Gradient 5% B for first 5 minutes, linear (1%) increase in B every minute afterwards).

        B. Multiple 2' site modifications
15   10 O.D. units of Oligomer 12 (Example 7-B) was treated with twice the amount of pyrene-1-butyric acid N-hydroxysuccinimide (6 mg in 400 μl DMF) and worked up in the same fashion as Example 23-A. Sephadex G-25 purification followed by HPLC purification gave the doubly pyrene-
20   conjugated oligonucleotide. The doubly conjugated oligonucleotide exhibited a HPLC retention time of 32.32 min. while the parent oligonucleotide had a retention time of 21.78 min. (Waters 600E with 991 detector; Hamilton PRP-1 column (15x25 cm); Solvent A: 50 mM TEAA, pH 7.0; B: 45 mM TEAA with
25   80% Acetonitrile; 1.5 mL/min. flow rate: Gradient 5% B for first 5 minutes, linear (1%) increase in B every minute afterwards).

     EXAMPLE 24
     Conjugation of Acridine at 2'-Position Of Oligonucleotides
30   A.   Single 2' site modification
        10 O.D. units (A_{260}) of Oligomer 12 (Example 7-B, about 60 nmols) was dried and dissolved in 1M $NaHCO_3$/Na2CO$_3$ buffer, pH 9.0, 200 μl. 9-acridinyl-isothiocyante, (5 mg, 2.1 μmols, Molecular Probes, Eugene, Oregon) was dissolved in 200 μl DMF.
35   This solution was added to the oligonucleotide, vortexed, covered with aluminum foil and left at 37°C overnight. The

reaction mixture was purified by passing through a Sephadex
G-25 column (1 x 40 cm) concentrated and further purified by
HPLC (reverse-phase). The product had a HPLC retention time
of 25.32 min. while the parent oligonucleotide had a retention
5　time of 21.78 min. (Waters 600E with 991 detector; Hamilton
PRP-1 column (15x25 cm); Solvent A: 50 mM TEAA, pH 7.0; B: 45
mM TEAA with 80% Acetonitrile; 1.5 mL/min. flow rate: Gradient
5% B for first 5 minutes, linear (1%) increase in B every
minute afterwards).

10　　　　B. Multiple 2' site modifications

　　　10 O.D. units (A_{260}) of Oligomer 13 (Example 7-B) in 400
μl of 1M Na2CO$_3$/NaHCO$_3$ buffer (pH 9.0) was treated with 10 mg
of 9-acridinyl-isothiocyanate in 400 μl of DMF. The reaction
mixture was vortexed, covered with aluminum foil and left at
15　37°C overnight. The reaction mixture was purified as for the
single site reaction of Example 24-A. The doubly conjugated
acridine-oligonucleotide eluted as the last peak in the HPLC
following single-modification products. The product had a
HPLC retention time of 32.32 min. while the parent oligo-
20　nucleotide had a retention time of 21.78 min. (Waters 600E
with 991 detector; Hamilton PRP-1 column (15x25 cm); Solvent
A: 50 mM TEAA, pH 7.0; B: 45 mM TEAA with 80% Acetonitrile;
1.5 mL/min. flow rate: Gradient 5% B for first 5 minutes,
linear (1%) increase in B every minute afterwards).

25　EXAMPLE 25

Conjugation Of Porphyrin at 2'-Position Of Oligonucleotides

　　　Methylpyroporphyrin XX1 ethyl ester (Aldrich) is
condensed with aminocaproic acid using N-hydroxysuccinimide
and EDAC. The resultant carboxylic acid is then activated
30　again with N-hydroxy succinimide and EDAC and treated with
Oligomer 12 as per the procedure of Example 23-A to give the
2'-porphyrin conjugated oligonucleotide.

2122030

EXAMPLE 26

Conjugation of Hybrid Intercalator-Ligand at 2'-Position Of Oligonucleotides

       A.   Photonuclease/intercalator ligand

5   The photonuclease/intercalator ligand 6-[[[9-[[6-(4-nitrobenzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoyl-pentafluorophenyl ester was synthesized as per the procedure of Egholm et al., J. Am. Chem. Soc. 1992, 114, 1895.

10   **B.   Single 2' site modification**

        10 O.D. units of Oligomer 12 (Example 7-B) was dissolved in 100 μl of 0.1 M borate buffer (pH 8.4) and treated with 330 μl of DMF solution (10 mg in 1 ml of DMF) of 6-[[[9-[[6-(4-nitrobenzamido)hexyl]amino]acridin-4-

15 yl]carbonyl]amino]hexanoylpentafluorophenyl ester. The solution was covered with aluminum foil and allowed to react overnight. The product was purified by Sephadex G-25 and HPLC purification of the reaction mixture.

       C.   Multiple 2' site modification

20   10 O.D. units A_{260} of Oligomer 13 (Example 7-B) was dissolved in 200 μl of 0.1 M borate buffer (pH 8.4) and treated with 660 μl of the DMF solution of 6-[[[9-[[6-(4-nitrobenzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoylpentafluorophenyl ester (10 mg in

25 1 ml solution) and the solution was covered with aluminum foil and left aside overnight. The bright yellow solution was purified by Sephadex G-25 and reverse phase HPLC to give the doubly conjugated oligonucleotide.

EXAMPLE 27

30 **Conjugation of Bipyridine Complex at 2'-Position Of Oligonucleotides**

       A.   Bipyridine complex

        Succinimidyl-4-carboxyl-4'-methyl-2,2'-bipyridine is synthesized according to the procedure of Telser, et al., J.

35 Am. Chem. Soc. 1989, 111, 7221.

54

B.   Single 2' site Modification

10 O.D. A_{260} units of Oligomer 12 is reacted with a 200
fold molar excess of succinimidyl-4-carboxyl-4'-methyl-2,2'-
bipyridine in 0.1 M borate buffer pH 8.5/DMF. The solution
5   is purified by Sephadex G-25 and reverse phase HPLC to yield
the conjugated oligonucleotide.

EXAMPLE 28
Conjugation of Aryl Azide Photocrosslinkers at 2'-Position Of
Oligonucleotides
10   **A.   Conjugation   of   N-hydroxysuccinimidyl-4-**
azidobenzoate (HSAB)
        Oligomer 14 (i.e., TTG CTT CCA* TCT TCC TCG TC wherein
A* represents 2'-O-pentyl amino adenosine, Example 7-C, 100
O.D. units, 595 nmols, based on the calculated extinction
15   coefficient of 1.6792 x 10^6 OD units) was dried and dissolved
in 500 ml of 0.2M NaHCO$_3$ buffer pH 8.1 and treated with 25 mg
of N-hydroxysuccinimidyl-4-azidobenzoate (HSAB, 96 μmols,
available both from Pierce & Sigma)) dissolved in 500 μl of
DMF. The reaction was allowed to react overnight at 37°C and
20   passed twice over Sephadex G-25 column (1 x 40 cm).   The
oligonucleotide fraction was purified by reverse-phase HPLC.
The product had the HPLC retention time of 38.79 min while the
parent oligonucleotide had the retention time of 33.69 min.
(5% → 40% CH3CN in 60 min.) in reverse phase column.
25   **B.   Conjugation   of   N-succinimidyl-6(4'-azido-2'-**
nitrophenyl-amino)hexanoate
        Oligomer 14 (Example 7-C, 200 OD units) was dissolved
in 500 ml NaHCO$_3$ buffer (0.2M, pH 8.1) and treated with 500 mg
of N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate
30   (SANPAH, 128 μmols, available both from Pierce and Sigma)
dissolved in 500 μl DMF. The reaction vial was wrapped with
aluminum foil and heated at 37°C overnight.   The reaction
mixture was passed twice over a Sephadex G-25 column (1 x 40
cm).   The oligonucleotide fraction was purified by reverse-
35   phase HPLC. The product had the HPLC retention time of 40.69
min. while the parent oligonucleotide had the retention time

2122030

of 33.69 min. (5% → 40% CH$_3$CN in 60 min.) in a reverse phase column.

PROCEDURE D

Duplex Melting Temperature of Conjugated Oligonucleotides

Utilizing the protocol described in Procedure B-1-b, the melting temperatures of various of the 2-aminolinked conjugate oligomers of the invention against a complementary DNA strand were obtained. The conjugate oligomers were compared to an oligomer of the same sequence bearing a 2'-O-pentylamino group. An un-modified, i.e., wild type, strand of the same sequence was also tested for comparison purposes. Both single site and multiple site conjugated oligomers were tested. As is shown in Table 5, the T$_m$ and the ΔT$_m$/modification, both as compared to 2'-pentylamino bearing oligomer, were measured. The wild type sequence is:

Oligomer 55:

CTG TCT CCA TCC TCT TCA CT

the single site sequence is:

Oligomer 12:

CTG TCT CCA* TCC TCT TCA CT

and the multiple site sequence is:

Oligomer 13:

CTG TCT CCA* TCC TCT TCA* CT

where A* represents a site of conjugation.

TABLE 5

Duplex Melting Temperature of
Conjugated Oligonucleotides Against DNA

Oligomer	Conjugate	$T_m \degree C$	ΔT_m/mod
55	wild type	60.5	--
12	2'-O-pentyl-NH$_2$	58.1	--
12	biotin	56.4	-1.7
12	cholic acid	55.5	-2.6
12	digoxigenin	55.8	-2.3
12	fluorescein	55.1	-3.0
12	pyrene	62.6	+4.5
12	acridine	58.6	+0.5
13	2'-O-pentyl-NH$_2$	56.9	--
13	biotin	54.4	-1.3
13	cholic acid	54.3	-1.3
13	digoxigenin	53.8	-1.6
13	fluorescein	53.4	-1.8
13	pyrene	65.1	+4.1
13	acridine	58.1	+1.2

EXAMPLE 29

Conjugation of Imidazole-4-acetic acid at 2'-Position of Oligonucleotides

    A. Activated imidazole-4-acetic acid

    Imidazole-4-acetic acid was reacted with 2,4-dinitrofluoro benzene. The resulting imidazole-N-(DNP)-4-acetic acid was converted to its N-hydroxy succinimide ester by treating with NHS/EDAC as per the procedure of Example 13.

    B. 2'-Site Modification

    10 O.D. A_{260} units of oligomer 12 was reacted with a 100 fold molar excess of imidazole-N-(DNP)-4-acetic acid NHS ester in 0.1M borate buffer pH 8.5/DMF (200 μL each). After overnight reaction, the reaction mixture was treated with 200 μL of mercaptoethanol to cleave off the DNP protecting group. The resulting reaction mixture was purified by passing through a Sephadex G-25 column followed by reverse phase HPLC to yield the imidazole conjugated oligonucleotide.

57 *2122030*

EXAMPLE 30

Conjugation of Metal Chelating Agents to 2'-Position
of Oligonucleotide

     A.   EDTA Complex

5   To form an EDTA Fe(II) complex for coupling to an
oligonucleotide as a nucleic acid cleaving agent, tricylohexyl
ester of EDTA is synthesized according to the procedure of
Sluka, et al., *J. Am. Chem. Soc.* **1990**, *112*, 6369.

     B.   EDTA 2'-Site Modification

10   The tricyclohexyl ester of EDTA (1.25 mg, 1.94 mmol)
and hydroxybenzotriazole (HOBt, 1 mg, 6.6 mmol) are dissolved
in DMF (50 μL) and EDAC 10 μL is added. To this solution,
oligonucleotide 12 (10 OD units) in 100 μL g 0.1M borate
buffer is added and left overnight. The solution is passed

15   through a Sephadex G-25 column and the oligonucleotide
fraction treated with conc. NH_3 (100 μL) for 1 hr. to cleave
off the acid protecting groups. Finally purification is
effected by size exclusion and HPLC.

     C.   DTPA 2'-Site Modification

20   Oligomer 12 was treated with diethylene triamine
pentaacetic anhydride (DTPA) in 0.1M $NaHCO_3$/DMF to offer
single-site modification. The conjugate was complexed with
Gadolinium ion (Gd III) to give a contrast agent, usable among
other uses, as an uptake measuring agent.

25 EXAMPLE 31

Conjugation   of   Cholesterol   To   The   2'-Position   of
Oligonucleotide

     A.   Method 1 - 2'-Aminolinker

     Cholesterol-hemisuccinate was converted to its N-

30 hydroxy succinimide ester. It was then conjugated to Oligomer
12 as per the procedure of Example 23-A or Oligomer 13 as per
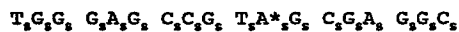the procedure of Example 23-B.

B. Method 2 - Conjugation of Cholesterol via a
Disulfide Bridge
Step 1

Thiocholesterol (1.4 g, 3.5 mmol) is added to a stirred
5 solution of 2,2'-dithiobis(5-nitropyridine) (1.4 g 4 mmol) in
chloroform 20 mL containing glacial acetic acid (400 μL) under
an argon atmosphere. The reaction is allowed to continue
overnight at room temperature, after which the precipitated
5-nitropyridine-2-thione was removed and the solution
10 evaporated and purified in a silica column to give S-(2-thio-
5-nitropyridyl)-thio cholesterol.
Step 2
Oligomer 55:

$T_sG_sG_s$ $G_sA_sG_s$ $C_sC_sG_s$ $T_sA^*_sG_s$ $C_sG_sA_s$ $G_sG_sC_s$

15 wherein $\overset{*}{A}$ represents an adenosine nucleotide funtionalized to
incorporate a 2'-O-pentylamino linking group is synthesized
as per Example 7. This oligonucleotide is then converted into
a thiol linker compound as per the procedure described for
Oligomer 50 in Example 20.
20   The thiol linker group containing oligonucleotide,
Oligomer 55, is reacted with an excess of S-(2-thio-5-
nitropyridyl)-thiocholesterol to conjugate the cholesterol
moiety to the 2' position of the oligonucleotide via a
disulfide bridge.

25 EXAMPLE 32
Synthesis of 2'-Aminolinker Containing Solid Supports for
DNA/RNA Synthesis
      A.   5'-Dimethyoxytrityl-2'-O-(pentyl-N-
phthalimido)uridine
30   5'-Dimethyoxytrityl-2'-O-(pentyl-N-phthalimido)uridine
was synthesized as per the procedure described in Example 18.
      B.   Succinate Nucleoside
      The nucleoside of step A (1 mmol) was treated with 4-
DMAP (122 mg, 1 mmol) and succinic anhydride (250 mg, 2.5
35 mmol) in 10 mL g anhydrous pyridine. After shaking overnight,
TLC (EtOAc: Hexane 6:4 with 0.1% Et_3N) indicated complete

2122030

succinylation of the nucleoside. 10 mL of water was added and the reaction shaken for an additional 1 hr. The reaction mixture was evaporated and partitioned between $CHCl_3$ and 20% citric acid (50 mL each). The chloroform layer was washed

5 with brine and evaporated. It was then used in the next step.

        C.   Nitrophenyl Succinate

           The dry 3'-O-succinate from step B was dissolved in dry dioxan (10 mL) containing pyridine (400 ml). 4-Nitrophenol (280 mg, 2 mmol) was added followed by DCC (1.32

10 g, 5 mmol) and the solution was shaken for 24 hrs. The fine precipitate of urea was filtered and the filtrate evaporated and applied to a silica column. The column was eluted with 5% CH_3OH in $CHCl_3$ containing 0.1% Et_3N. The 3'-nitrophenyl succinate of the nucleoside eluted first from the column. The

15 product containing fractions were evaporated to give a foam.

        D.   Nucleoside Solid Support

           The 3'-nitrophenyl succinate from step C was dissolved in 5 mL of anhydrous DMF and treated with 5g of 500 ˚A pore diameter aminopropyl CPG support and shaken for 8 hrs. The

20 CPG support was filtered, washed with methanol (5 x 20 ml) followed by ether (5 x 20) and dried. The CPG support was capped by treating for 30 min. with pyridine/acetic anhydride/N-methyl imidazole (20 ml, 8:1:1 v/v/v). The CPG support was then filtered off, washed with pyridine, methanol

25 and ether and air-dried. Assay of the dimethoxy trityl showed the loading capacity of the CPG support was 27 mmols/gram.

PROCEDURE E

Determination of Cellular Uptake of Folic Acid Conjugated Oligonucleotide

30   The effect of conjugation of an oligonucleotide with folic acid was determined by the inhibition of ICAM-1 utilizing the method of Chiang, et al., J. Biol. Chem. 1991, 266, 18162. Utilizing this method, human lung epithelial carcinoma cells (A549 cells) were grown to confluence in 96

35 well plates. Medium was removed and the cells were washed with folic acid free medium three times. Folic acid free

60

medium was added to the cells and increasing concentrations
of an ICAM-1 specific antisense phosphorothioate
oligonucleotide having the sequence 5'-TGG GAG CCA TAG CGA
GGC-3', either free or conjugated to folic acid, was added to
5   the incubation medium.   This oligonucleotide is an 18 base
phosphorothioate oligonucleotide that targets the AUG
translation initiation codon of the human ICAM-1 mRNA (Chiang
et al., J. Biol. Chem. 1991, 266, 18162).   The
oligonucleotides were incubated with the A549 cells for 24
10 hours then ICAM-1 was induced by adding 2.5 ng/ml tumor
necrosis factor-α to the medium.   Cells were incubated an
additional 15 hours in the presence of tumor necrosis factor-α
and oligonucleotide.   ICAM-1 expression was determined by a
specific ELISA as described by Chiang, et al..   We had
15 previously demonstrated that the addition of the test
oligonucleotide to incubation medium alone does not result in
inhibition of ICAM-1 expression. However formulation of the
test oligonucleotide with cationic liposomes results in at
least a 1000 fold increase in potency and also correlates with
20 the appearance of the oligonucleotide in the nucleus (Bennett,
et al., Molecular Pharmacology 1991, 41, 1023).   The results
of this test are shown in Table 6.   At the 30uM level, the
folic acid conjugated oligonucleotide shows an approximate 40%
enhancement in activity.

25   TABLE 6

                           ICAM-1 Activity

     Concentration   Oligonucleotide   Oligonucleotide +
                            Control   Folic Acid - Percent
                                                   Of Control
30   ──

     1 uM   105.1   107.6
     3 uM   112.0   104.4
     10 uM   112.4   92.9
     30 uM   108.4   61.6

61

Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred embodiments of the invention and that such changes and modifications may be made without departing from the spirit

5 of the invention. It is therefore intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

WE CLAIM:

1. A compound comprising a plurality of linked nucleosides, wherein at least one of said nucleosides is functionalized at its 2'-position by attachment of a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, a hybrid photonuclease/intercalator, or an aryl azide photo-crosslinking agent.

2. The compound of claim 1 wherein a linking moiety attaches said steroid molecule, reporter molecule, non-aromatic lipophilic molecule, reporter enzyme, peptide or protein to the 2'-position of said functionalized nucleoside.

3. The compound of claim 1 wherein said functionalized nucleoside comprises a steroid molecule, biotin, a fluorescein dye molecule, an alicyclic hydrocarbon molecule, a saturated or unsaturated fatty acid, a wax, a terpene, a polyalicyclic hydrocarbon, adamantane, a buckminsterfullerene, a reporter enzyme, a peptide, a protein, thiamine, riboflavin, nicotinic acid, niacin, pyridoxal, pantothenic acid, biotin, folic acid, methyl folate, a vitamin B_{12} cobamide coenzyme, inositol, choline, ascorbic acid, retinoic acid, retinol, a tocopherol, vitamin D, vitamin K, EDTA, DTPA, o-phenanthroline, a photonuclease/intercalator, an aryl azide photo-crosslinker, a phenanthroline/metal complex and a bipyridine/metal complex attached to the 2'-position of said nucleoside.

4. The compound of claim 1 wherein said functionalized nucleoside comprises a steroid molecule attached via a linking moiety to the 2'-position of said nucleoside.

2122030

5.   The compound of claim 2 wherein said linking moiety comprises an Ω-aminoalkoxy or Ω-aminoalkylamino moiety.

6.   The compound of claim 5 wherein said linking moiety comprises an Ω-aminoalkoxy moiety.

7.   The compound of claim 1 wherein said functionalized nucleoside comprises a reporter enzyme, a peptide or protein attached via a linking moiety to the 2'-position of said nucleoside.

8.   The compound of claim 1 wherein cholic acid, deoxycholic acid, dehydrocholic acid, cortisone, digoxigenin, testosterone, cholesterol or digoxigehin is attached at the 2' position of said nucleoside.

9.   The compound of claim 8 wherein attachment is through a linking moiety comprises an Ω-aminoalkoxy or Ω-aminoalkylamino moiety.

10.   The compound of claim 9 wherein said linking moiety comprises an Ω-aminoalkoxy moiety.

11.   The compound of claim 10 wherein said linking moiety comprises a 5-aminopentoxy group.

12.   The compound of claim 1 wherein cholic acid is attached at the 2' position of said nucleoside.

13.   The compound of claim 12 wherein said attachment is through a linking moiety comprises an Ω-aminoalkoxy or Ω-aminoalkylamino moiety.

14.   The compound of claim 13 wherein said linking moiety comprises an Ω-aminoalkoxy moiety.

15. The compound of claim 14 wherein said linking moiety comprises a 5-aminopentoxy group.

16. The compound of claim 1 wherein at least one of the linked nucleosides is a 2'-deoxy'-2'-fluoro, 2'-methoxy, 2'-ethoxy, 2'-propoxy, 2'-aminoalkoxy or 2'-allyloxy nucleoside.

17. The compound of claim 1 wherein at least two of the linked nucleosides are linked by phosphorothioate linking groups.

18. A compound comprising a plurality of linked nucleosides, wherein at least one of the nucleosides is a functionalized nucleoside selected from the group consisting of:
a 2' functionalized nucleoside having cholic acid linked to the 2' position of the nucleoside;
a heterocyclic base functionalized nucleoside having cholic acid linked to the heterocyclic base of the nucleoside;
a 5' terminal nucleoside having cholic acid linked to the 5'-position of the nucleoside;
a 3' terminal nucleoside having cholic acid linked to the 3'-position of the nucleoside; and
an inter-strand nucleoside having cholic acid linked to an inter-nucleotide linkage linking said inter-strand nucleoside to an adjacent nucleoside.

19. The compound of claim 18 wherein a linking group links said cholic acid and the functionalized nucleoside.

20. The compound of claim 18 wherein said functionalized nucleoside is a 2' functionalized nucleoside having cholic acid linked to the 2'-position of the nucleoside.

21. The compound of claim 18 wherein at least one of the linked nucleosides is a 2'-deoxy'-2'-fluoro, 2'-methoxy, 2'-ethoxy, 2'-propoxy, 2'-aminoalkoxy or 2'-allyloxy nucleoside.

22. The compound of claim 18 wherein at least two of the linked nucleosides are linked by phosphorothioate linking groups.

23. The compound of claim 18 wherein said functionalized nucleoside is a 3' terminal nucleoside having cholic acid linked to the 3'-position of said 3' terminal nucleoside.

24. The compound of claim 18 wherein said functionalized nucleoside is a 5' terminal nucleoside having cholic acid linked to the 5'-position of said 5' terminal nucleoside.

25. The compound of claim 18 wherein said functionalized nucleoside is a heterocyclic base functionalized nucleoside having cholic acid linked to the heterocyclic base of said nucleoside.

26. The compound of claim 18 wherein said functionalized nucleoside is an inter-strand nucleoside having cholic acid linked to an inter-nucleotide linkage linking said inter-strand nucleoside to an adjacent nucleoside.

27. A compound comprising a plurality of linked nucleosides, wherein:

at least one of the nucleosides is a 2'-deoxy'-2'-fluoro, 2'-O-C_1-C_{20}-alkyl, 2'-O-C_2-C_{20}-alkenyl, 2'-O-C_2-C_{20}-alkynyl, 2'-S-C_1-C_{20}-alkyl, 2'-S-C_2-C_{20}-alkenyl, 2'-S-C_2-C_{20}-alkynyl, 2'-NH-C_1-C_{20}-alkyl, 2'-NH-C_2-C_{20}-alkenyl, 2'-NH-C_2-C_{20}-alkynyl nucleoside; and

at least one of the nucleosides is a functionalized
nucleoside selected from the group comprising::

a 2'-functionalized nucleoside having a steroid
molecule, a reporter molecule, a non-aromatic
lipophilic molecule, a reporter enzyme, a peptide, a
protein, a water soluble vitamin, a lipid soluble
vitamin, an RNA cleaving complex, a metal chelator, a
porphyrin, an alkylator, a hybrid photonuclease/inter-
calator, or an aryl azide photo-crosslinking agent
linked to the 2'-position of the nucleoside;

a heterocyclic base functionalized nucleoside
having a steroid molecule, a reporter molecule, a non-
aromatic lipophilic molecule, a reporter enzyme, a
peptide, a protein, a water soluble vitamin, a lipid
soluble vitamin, an RNA cleaving complex, a metal
chelator, a porphyrin, an alkylator, a hybrid photo-
nuclease/intercalator, or an aryl azide photo-
crosslinking agent linked to the heterocyclic base of
the nucleoside;

a 5' terminal nucleoside having a steroid
molecule, a reporter molecule, a non-aromatic
lipophilic molecule, a reporter enzyme, a peptide, a
protein, a water soluble vitamin, a lipid soluble
vitamin, an RNA cleaving complex, a metal chelator, a
porphyrin, an alkylator, a hybrid photonuclease/inter-
calator, or an aryl azide photo-crosslinking agent
linked to the 5'-position of the nucleoside;

a 3' terminal nucleoside having a steroid
molecule, a reporter molecule, a non-aromatic
lipophilic.molecule, a reporter enzyme, a peptide, a
protein, a water soluble vitamin, a lipid soluble
vitamin, an RNA cleaving complex, a metal chelator, a
porphyrin, an alkylator, a hybrid photonuclease/inter-
calator, or an aryl azide photo-crosslinking agent
linked to the 3'-position of the nucleoside; and

an inter-strand nucleoside having a steroid
molecule, a reporter molecule, a non-aromatic

2122030

lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, a hybrid photonuclease/intercalator, or an aryl azide photo-crosslinking agent linked to an inter-stand linkage linking said inter-strand nucleoside to an adjacent nucleoside.

28. The compound of claim 27 wherein:
said functionalized nucleoside is a nucleoside having a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, a hybrid photonuclease/intercalator, or an aryl azide photo-crosslinking agent linked to the 2'-position of said nucleoside; and

the remainder of said linked nucleosides are 2'-deoxy-2'-fluoro, 2'-methoxy, 2'-ethoxy, 2'-propoxy, 2'-aminoalkoxy or 2'-allyloxy nucleosides.

29. The compound of claim 27 wherein said functionalized nucleoside is a 3' terminal nucleoside having a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, a hybrid photonuclease/intercalator, or an aryl azide photo-crosslinking agent linked to the 3'-position of the nucleoside.

30. The compound of claim 27 wherein said functionalized nucleoside is a 5' nucleoside having a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator, a hybrid

photonuclease/intercalator, or an aryl azide photo-
crosslinking agent linked to the 5'-position of the
nucleoside.

31.   The compound of claim 27 wherein said functionalized
nucleoside is a heterocyclic base functionalized nucleoside
having a steroid molecule, a reporter molecule, a non-aromatic
lipophilic molecule, a reporter enzyme, a peptide, a protein,
a water soluble vitamin, a lipid soluble vitamin, an RNA
cleaving complex, a metal chelator, a porphyrin, an alkylator,
a hybrid photonuclease/intercalator, or an aryl azide photo-
crosslinking agent linked to the heterocyclic base of the
nucleoside.

32.   The compound of claim 27 wherein said functionalized
nucleoside is an inter-strand nucleoside having a steroid
molecule, a reporter molecule, a non-aromatic lipophilic
molecule, a reporter enzyme, a peptide, a protein, a water
soluble vitamin, a lipid soluble vitamin, an RNA cleaving
complex, a metal chelator, a porphyrin, an alkylator, a hybrid
photonuclease/intercalator, or an aryl azide photo-
crosslinking agent linked to an inter-strand linkage linking
said nucleoside to an adjacent nucleoside.

33.   The compound of claim 27 wherein at least two of the
linked nucleosides are linked by phosphorothioate linking
groups.

34.   Use of a compound having a plurality of linked nucleosides, wherein said compound includes at least one nucleoside functionalized at its 2'-position by attachment of a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, or a lipid soluble vitamin to the 2'-position with a linking moiety to effect cellular uptake of said compound in an organism.

35.   The use of claim 34 further including selecting said steroid molecule from the group consisting of cholic acid, deoxycholic acid, dehydrocholic acid, cortisone, digoxigenin, testosterone, cholesterol and digoxigenin.

36.   The use of claim 34 further including selecting cholic acid as said steroid molecule.

37.   The use of claim 34 further including selecting an Ω-aminoalkylamino moiety as said linking molecule.

38.   A method of derivatizing an oligonucleotide comprising:

    functionalizing said oligonucleotide with at least one of a steroid molecule, a reporter molecule, a non-aromatic lipophilic molecule, a reporter enzyme, a peptide, a protein, a water soluble vitamin, a lipid soluble vitamin, an RNA cleaving complex, a metal chelator, a porphyrin, an alkylator,

A

69a

a hybrid photonuclease/intercelator, or an aryl azide photo-crosslinking agent; and

further functionalizing said oligonucleotide with one of a group to effect increased affinity of said oligonucleotide for a target or a group to increase the stability of said oligonucleotide to nucleases.

39. The method of claim 38 wherein said oligonucleotide is functionalized with one of a steroid molecule, biotin, a fluorescein dye molecule, an alicyclic hydrocarbon molecule, a saturated or unsaturated fatty acid, a wax, a terpene, a polyalicyclic hydrocarbon, adamantane, a buckminster-fullerene, a reporter enzyme, a peptide, a protein, pyrene, thiamine, riboflavin, nicotinic acid, niacin, pyridoxal pantothenic acid, biotin, folic acid, methyl folate, a vitamin b_{12} cobamide coenzyme, inositol, choline, ascorbic acid, retinoic acid, retinol, a tocopherol, vitamin d, vitamin K, EDTA, DTPA, o-phenanthroline, a photonuclease/intercalator, a bipyridine complex, an aryl

azide photo-crosslinker, a phenanthroline/metal complex or a
bipyridine/metal complex.

40. The method of claim 38 including further
functionalizing said oligonucleotide to both increase affinity
of said oligonucleotide for a target and to increase the
stability of said oligonucleotide to nucleases.

41. A compound comprising a plurality of linked
nucleosides, wherein at least one of the nucleosides is a
functionalized nucleoside selected from the group consisting
of:
        a 2' functionalized nucleoside having folic acid linked
to the 2' position of the nucleoside;
        a heterocyclic base functionalized nucleoside having
folic acid linked to the heterocyclic base of the nucleoside;
        a 5' terminal nucleoside having folic acid linked to
the 5'-position of the nucleoside;
        a 3' terminal nucleoside having folic acid linked to
the 3'-position of the nucleoside; and
        an inter-strand nucleoside having folic acid linked to
an inter-nucleotide linkage linking said inter-strand
nucleoside to an adjacent nucleoside.

42. A compound of claim 41 wherein said functionalized
nucleoside is a 2' functionalized nucleoside having folic acid
linked to the 2' position of the nucleoside.

43. A compound comprising a plurality of linked
nucleosides, wherein at least one of the nucleosides is a
functionalized nucleoside selected from the group consisting
of:
        a 2' functionalized nucleoside having pyrene linked to
the 2' position of the nucleoside;
        a 5' terminal nucleoside having pyrene linked to the
5'-position of the nucleoside;

a 3' terminal nucleoside having pyrene linked to the
3'-position of the nucleoside; and

an inter-strand nucleoside having pyrene linked to an
inter-nucleotide linkage linking said inter-strand nucleoside
to an adjacent nucleoside.

44.   A compound of claim 43 wherein said functionalized
nucleoside is a 2' functionalized nucleoside having pyrene
linked to the 2' position of the nucleoside.